Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8271–8274, November 1989
Biochemistry

Effect of 3′ flanking neighbors on kinetics of pairing of dCTP or dTTP opposite O^6-methylguanine in a defined primed oligonucleotide when *Escherichia coli* DNA polymerase I is used

(fidelity/mutation/base pairing/*Drosophila* DNA polymerase α)

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Communicated by Heinz Fraenkel-Conrat, July 27, 1989

ABSTRACT **O^6-Methylguanine (m^6G) was incorporated site-specifically into two 25-base oligonucleotides differing only in the nucleotide on the 3′ side of the modified base. Templates were primed with oligonucleotides terminating one or two bases prior to the site at which incorporation kinetics were to be investigated. *Escherichia coli* DNA polymerase I (Klenow fragment) was used to determine the apparent K_m and relative V_{max} of incorporation of either dCTP or dTTP opposite m^6G or G. These data were used to calculate the relative frequency of incorporation opposite the m^6G or the unmodified G. When the sequence was 3′-Cm^6G-5′, there was a 6- to 7-fold preference for formation of a m^6G·T pair compared with m^6G·C. The m^6G·T frequency, based on V_{max}/K_m, was at least 50-fold greater than that of a G·T pair at the same site. Changing the sequence to 3′-Tm^6G-5′ had a marked effect on both K_m and V_{max} of pairs containing m^6G and on the incorporation frequency of T opposite m^6G, which was then only slightly favored over m^6G·C. When replication was started directly opposite m^6G, the kinetics appeared unaffected. These data indicate that the frequency of incorporation of C or T opposite m^6G in a DNA template is dependent on the flanking neighbors and that a change of even a single base at the 3′ position can have a major effect on mutagenic efficiency. Replication using *Drosophila* Pol α gave the same values for relative frequencies. Pairing of either C or T with m^6G on the primer terminus did not significantly inhibit extension of the next normal base pair, in contrast to terminal mismatches of unmodified bases. It is concluded that, in the absence of repair, m^6G can exhibit widely differing mutation frequencies which, in these experiments, can be as high as 85% of the replicated base. This variation in frequency of changed pairing could contribute to the occurrence of mutational "hot spots" after replication of damaged DNA.**

The likely role of O^6-alkylguanines as a major factor in mutagenesis by certain alkylating agents was recognized by Loveless about 20 years ago (1). In an attempt to resolve contradictions in the literature, he suggested that O^6-methylguanine (m^6G) might pair with thymine (T) during replication and thus cause the G·C → A·T transitions found as the major genetic change after reaction with certain carcinogenic alkylating agents. Further studies in a number of laboratories established that the occurrence, and in particular the persistence of this alkylated base, often correlated with the biological endpoints of mutagenesis and carcinogenesis (2). In accord with the proposed mechanism of mutagenesis, m^6G can pair with T both *in vitro* (3) and *in vivo* (4), when presented to a polymerase either in the template or as a precursor to DNA synthesis.

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This model of mutagenesis occurring by base pairing between m^6G and T during replication has long been assumed, but only recently has it begun to be tested rigorously [reviewed by Basu and Essigmann (5)]. When an m^6G-containing dodecamer was annealed with a series of analogous oligomers containing A, G, T, or C opposite the m^6G site, the thermal stabilities were all greatly decreased (6). Changing nearest neighbors of m^6G, using a series of nonamers, again revealed low melting temperatures regardless of sequence (7). The most stable pair, m^6G·C, could arise by protonation of the N-3 of C (8), but the range of duplex stabilities lay in the middle of the range of normal mismatches.

The use of ^{1}H-NMR analysis of the duplex oligonucleotides, in which m^6G was positioned opposite each of the four possible pairing partners, did indicate that formal hydrogen bonding, at least as shown by this technique, did not explain why replication by polymerases showed a definite preference to pair the adduct with T (3). In contrast, ^{31}P-NMR analysis showed that the m^6G·T pair had almost undetectable architectural distortion of the phosphodiester backbone, whereas all other pairings (including m^6G·C) displayed significant deviations from the normal structure (9, 10).

While informative, none of these types of experiments can yield quantitative kinetic data or the mutation frequency of a single m^6G in a defined template. In the present paper we have constructed two 25-mer templates with m^6G at position 19, flanked on the 3′ side by either C or T. With a 5′-^{32}P-end-labeled 17-mer or 18-mer as primer, a gel extension assay (11) was used to measure the effect of m^6G on the kinetics of extension by the Klenow fragment of *Escherichia coli* DNA polymerase I. This approach showed the high preference for pairing of T versus C when the 3′ neighbor was C or a C·G pair. When T or T·A replaced C or C·G in the template-primer complex, this preference was greatly reduced. Such neighbor effects have been hypothesized from *in vivo* experiments showing nonrandom mutation (12–14). The specificity of the Ha-*ras*-1 locus activation by methylnitrosourea also points to the importance of sequence. The observed G → A point mutation (codon 12) is believed to arise through formation of m^6G and its subsequent pairing with T (15). Our data furnish a quantitative basis for these observations, and we suggest that relatively small conformational changes are critical in mutation.

EXPERIMENTAL

Materials. Cloned Klenow fragment of *E. coli* DNA polymerase I, as well as the dNTP substrates (HPLC-purified),

Abbreviations: m^6G, O^6-methylguanine; Pol α, DNA polymerase α.

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was purchased from Pharmacia. Purified *Drosophila melanogaster* DNA polymerase α (Pol α) consisting of at least three polypeptide subunits, including primase (16), was a generous gift of I. R. Lehman (Stanford University). Solvents and other reagents of the highest purity for the oligonucleotide synthesis were purchased from standard suppliers. Snake venom phosphodiesterase was purchased from Sigma; bacterial alkaline phosphatase was from New England Biolabs. The [γ-^{32}P]ATP was obtained from New England Nuclear and the T4 polynucleotide kinase was obtained from United States Biochemical.

Oligonucleotide Synthesis. Oligonucleotides were synthesized on a 1-μmol scale on an Applied Biosystems model 381A automated synthesizer by the cyanoethylphosphoramidite method (17, 18). The oligonucleotides containing m^{6}G were prepared from the protected monomer 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-*N*^{2}-isobutryl-*O*^{5}-methylguanosine 3′-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite], purchased from American Bionetics (Emeryville, CA). The only deviation from the published synthesis protocol was during the deprotection stage, when the nonaqueous conditions of 1,8-diazabicyclo[5.4.0]undec-7-ene in tetrahydrofuran and methanol were employed (19, 20). The prolonged (2 weeks at room temperature) deprotection with this reagent, rather than ammonia, was employed to ensure complete removal of protecting groups and to avoid side reactions such as conversion of m^{6}G to 2,6-diaminopurine (19). The following deoxyoligonucleotides were synthesized: 5′-CCGCTAGCGGGTACCG-AGCTCGAAT-3′ (called G-1 or m^{6}G-1) and 5′-CCGCTAG-TGGGTACCGAGCTCGAAT-3′ (called G-2 or m^{6}G-2), in which the underlined bases (position 19) were either G or m^{6}G; the 17-mer 3′-CCCATGGCTCGACTTA-5′; and the two 18-mers 3′-GCCCATGGCTCGACTTA-5′ and 3′-TCCCATGGC-TCGACTTA-5′.

Oligonucleotide Purity. In addition, the hexamer 5′-GCTAm^{6}GC-3′ was synthesized by standard methods as described above. After deprotection, the hexamer was desalted by using a Sephadex G-10 column (2.5 × 40 cm) eluted with water. All oligonucleotides were further purified by electrophoresis on 20% polyacrylamide gels; they were visualized with UV light, excised, and finally eluted in water. The hexamer was subjected to reverse-phase HPLC analysis [0–50% (vol/vol) CH_3CN in 0.1 M ammonium acetate (pH 6.8) over 45 min at 1 ml/min] on a Beckman 322 gradient liquid chromatograph equipped with a Hewlett–Packard 1040A diode array detector. In parallel the samples were 5′-phosphorylated by using polynucleotide kinase and [γ-^{32}P]ATP (21) and again subjected to electrophoresis on a 20% polyacrylamide gel followed by autoradiography to check the purification steps.

The nucleoside composition of the purified m^{6}G-containing hexamer was assessed by HPLC analysis after complete digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. Nucleosides were separated by reverse-phase HPLC using a 5-μm Ultrasphere ODS column (Beckman) eluted at 0.75 ml/min with a gradient over 45 min of 0–35% CH_3CN in 0.1 M ammonium acetate (pH 6.8). The nucleotide composition for the hexamer, determined by integration with the diode array, was found to be within the theoretical composition for GCTAm^{6}GC. All UV spectra of the nucleosides had the correct λ_{max}, λ_{min}, and UV ratios.

This hexamer, and its control containing unmodified G, can be inserted into a unique site in M13 DNA for replication and repair studies.

Incorporation and Extension Using DNA Polymerase I (Klenow Fragment) or *Drosophila* Pol α. The ^{32}P-5′-end-labeled primers annealed with the two templates were used in a gel assay, following the procedures described by Boosalis *et al.* (11) and Singer *et al.* (22). Reactions for kinetic data were carried out at 37°C for 90 sec with the Klenow fragment and for 4 min with Pol α. The gel electrophoresis, autoradiography, densitometry, and data analysis were done as described by Boosalis *et al.* (11). Time course experiments were done by using the same procedures, except that the times were 30 sec to 10 min. For DNA templates with C 3′ to G or m^{6}G, 10 μM dGTP was present with various concentrations of dCTP or dTTP. For DNA templates with T 3′ to G or m^{6}G, 10 μM dATP replaced the dGTP.

RESULTS AND DISCUSSION

The major aim of this experiment was to investigate the mutagenic potential of the carcinogenic analog m^{6}G by comparing the relative incorporation of T versus C opposite m^{6}G or G in a template. Klenow fragment and *Drosophila* Pol α were used to copy the aberrant template site. These measurements were carried with two templates differing only by the identity of the base 3′ to m^{6}G (see *Experimental*). The kinetics of primer extension were measured by PAGE.

In any kinetic analysis a primary concern should be the purity of the template and incoming dNTP. With this in mind, we rigorously purified the templates containing m^{6}G, as well as the unmodified base and the primer oligonucleotides. The templates contained G and m^{6}G in appropriate molar ratios. In addition, the hexamer 5′-GCTAGm^{6}GC-3′, which was also synthesized and characterized prior to synthesis of the 25-mer, was free of any detectable contaminant. This was assessed for both oligonucleotides by HPLC separation of the nucleosides after enzyme digestion, followed by spectral analysis of the products.

Fig. 1 and 2 illustrate the gel extension method using one of the templates, that with C 3′ to the G or m^{6}G. The Hanes–Woolf plots for both templates (3′ C and 3′ T) in a single experiment are shown in Fig. 3. Such data enable us to determine kinetic parameters apparent K_m (K_m^{app}) and relative V_{max} (V_{max}^{rel}) for incorporation of any dNMP opposite any base (app indicates apparent). This method has been used in other laboratories to answer questions of polymerase specificity

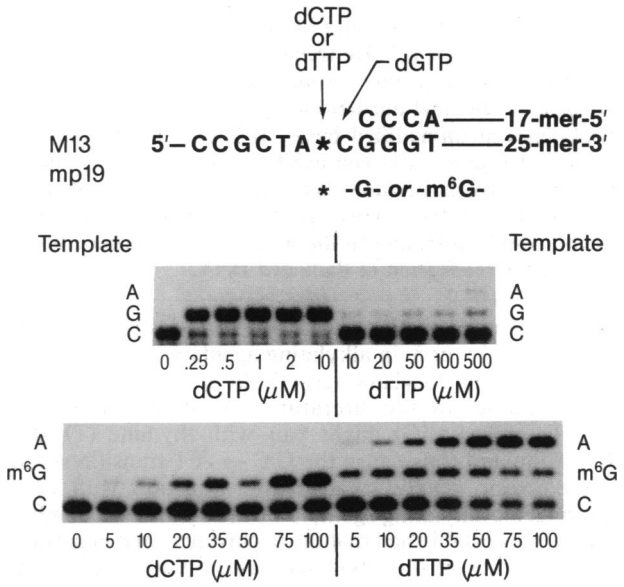


FIG. 1. Illustration of gel electrophoresis used to separate extended primers. The primer band is not shown. Reactions were for 90 sec using the Klenow fragment, with increasing molarities of the indicated dNTP, in the presence of 10 μM dGTP to form the initial C·G pairs. The templates used are shown at the top. Note on the lower right side the incorporation of dTTP opposite m^{6}G, with extension to form an A·T pair. Kinetic data are in Table 1 and representative Hanes–Woolf plots for this oligomer, m^{6}G-1, are in Fig. 3.

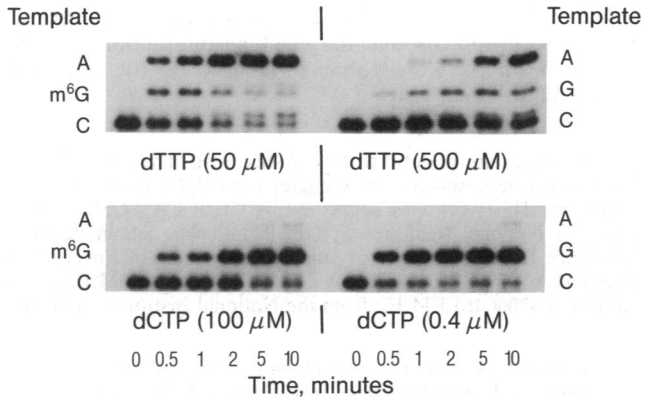


FIG. 2. Illustration of the time course of replication, when oligomer m^{6}G-1 and its G-containing 25-mer primed with the complementary 17-mer were used. All reaction mixtures contained 10 μM dGTP with dTTP and dCTP concentrations at approximately the K_m^{app}. The primer band is not shown. The control oligomer, with G in position 19, is on the right side. The G·T pair can be formed, particularly with long incubations, and extension to the 20-mer, by formation of A·T, is rapid. The upper left shows almost complete extension of the 18-mer, by formation of C·G, as well as rapid formation of the 19-mer by m^{6}G·T pairing and even more rapid extension to the 20-mer by A·T pairing. The doublets seen after 2 min may arise from a small amount (1–2%) of contaminating 16-mer primer forming a G·T pair, which can extend (see upper right) and has a different mobility from a G·C-containing oligonucleotide. This mobility difference can be seen in the lower part of Fig. 1, where m^{6}G·T-containing oligonucleotide migrates slower than that containing m^{6}G·C at the terminus.

and fidelity for mismatches involving unmodified bases (23–26). In our laboratory we applied it to a study of the kinetics of inserting O^2- and O^4-alkyl-dTTP opposite A (22).

In Fig. 1 it should be noted that the wobble pair G·T is not formed readily, and when it occurs it is a block to extension to the next pair, which would be A·T. The effect of a mismatch on extension to a normal base pair has been previously reported as greatly hindering replication (23). In contrast, when m^{6}G is in the template, at the same concentration of dTTP (e.g., 35 μM) there is both formation of the m^{6}G·T pair and rapid extension to the A·T pair.

The incorporation of dCMP is less favored than incorporation of dTMP opposite m^{6}G. In the time course shown in the lower part of Fig. 2, extension of both m^{6}G·C and G·C to an A·C mispair can barely be detected after 10 min. Extension of the m^{6}G·T terminus to an A·T base pair is almost complete by this time (upper left). The specificity for inserting dNTP substrates is governed by V_{max}^{rel}/K_m^{app} (11). The ratio f of V_{max}^{rel}/K_m^{app} for "wrong" (w) compared to "right" (r) substrates, Eq. **1**, corresponds to the ratio of wrong to right incorporation.

$$f = \frac{(V_{max}^{rel}/K_m^{app})_w}{(V_{max}^{rel}/K_m^{app})_r}. \quad [1]$$

Individual measurements of K_m and V_{max} are not required to determine fidelity ($1/f$); only the V_{max}^{rel}/K_m^{app} ratios are needed. Accurate estimates of V_{max}^{rel}/K_m^{app} are obtained from the intercept of a Hanes–Woolf plot, [dNTP]/v as a function of [dNTP].

The efficiency of misincorporating dCMP or dTMP, relative to a normal pair in each template, is given in Tables 1 and 2. In Table 1 results are shown from two different primers annealed with the same template. In one case the 17-mer is extended by the polymerase to a G·C pair before incorporation of the dNMP opposite m^{6}G or G is measured. In the second case we annealed an 18-mer to form this initial G·C pair. Both primers, within experimental error, gave the same

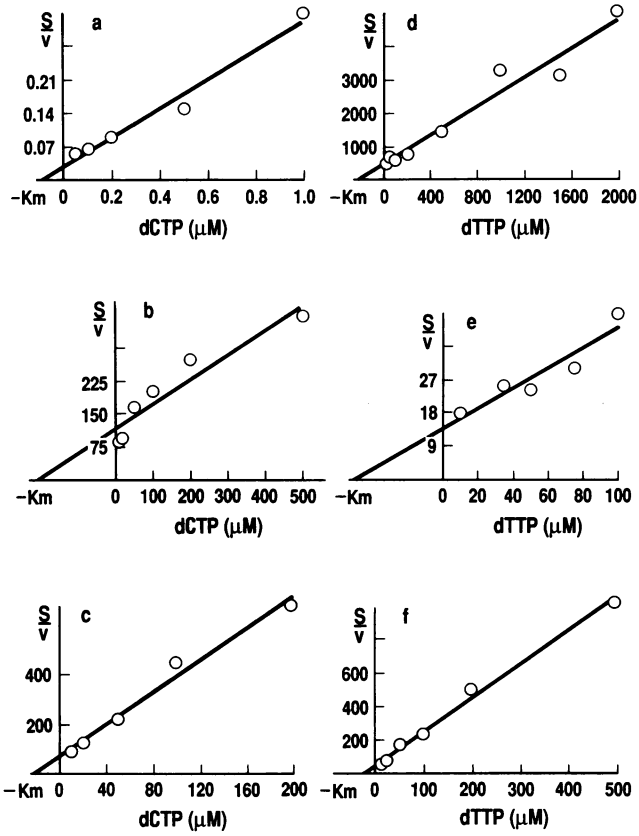


FIG. 3. Representative Hanes–Woolf plots used to determine K_m^{app} and V_{max}^{rel} for nucleotide insertion opposite G or m^{6}G. Plots *a* and *d* are for control 25-mer, primed with the 17-mer. Plots *b* and *e* are for m^{6}G-1 (C 3′ to m^{6}G). Plots *c* and *f* are for m^{6}G-2 (T 3′ to m^{6}G). Averaged kinetic data from such experiments are given in Tables 1 and 2.

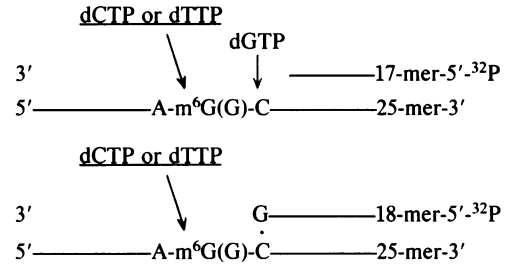
results. It is apparent that the G·T wobble pair is the least favored possible pair in this series. The m^{6}G·C pair, which has a K_m^{app} similar to that of G·T, is formed with a much higher relative V_{max}^{rel} so that its rate of formation is about 8 times greater. m^{6}G·T has a high V_{max}^{rel} and a relatively low K_m^{app}, thus favoring this pairing by about 50-fold over a G·T pair. More important, however, is that the mutagenic event m^{6}G·T formation, which leads to a transition, is about 7-fold favored over m^{6}G·C formation, which would not cause a point mutation.

In preliminary experiments using *Drosophila* Pol α, containing no detectable 3′→5′ exonuclease activity (27), we found a high preference for the formation of m^{6}G·T pairs compared to G·T pairs when templates m^{6}G-1 and G-1 were used. In agreement with data in Table 1, Pol α prefers m^{6}G·T formation to m^{6}G·C formation. This preference is not observed when m^{6}G-2 and G-1 are the templates (data not shown).

When a single base 3′ to m^{6}G or G is changed from C to T in an otherwise identical sequence, there is a major change in the kinetic parameters for all but the normal Watson–Crick G·C pair. This is shown in both Table 2 and the Hanes–Woolf plots (from a single series) in Fig. 3. Fig. 3 *b* and *c* compare dCTP insertion opposite m^{6}G and Fig. 3 *e* and *f* compare dTTP opposite m^{6}G. Although both K_m and V_{max} differ in the two templates, calculation of misinsertion ratio of f shows that only the m^{6}G·T pair has a changed and much lower (5- to 6-fold) f value. Thus, the mutagenic potential of an m^{6}G 3′ to T may be at least 5-fold lower than when adjacent to C.

Again we address the question of purity of the templates. This is particularly important for the templates in Table 1, where the V_{max}^{rel} with the incorporation of dCMP into the

Table 1. Kinetics of incorporation of dTTP or dCTP opposite m^{6}G 3′ to C in M13 DNA fragment m^{6}G-1



Pair	K_m^{app}, μM	V_{max}^{rel}	f
G·C (3)	0.16 ± 0.06	3.1 ± 0.7	1
G·T (3)	264 ± 181	0.4 ± 0.1	0.8×10^{-4}
m^{6}G·C (4)	233 ± 29	3.0 ± 1.4	6.7×10^{-4}
m^{6}G·T (5)	43 ± 10	3.7 ± 0.8	44×10^{-4}

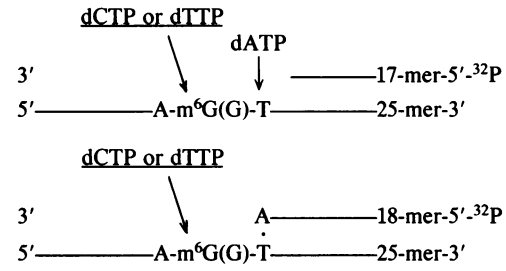
The number of independent determinations is shown in parentheses. The SEM is given for each value. f is the ratio of each misincorporation efficiency compared to that for formation of the normal base pair, G·C (see Eq. 1). The kinetic constants were derived from 90-sec incubation times to fulfill the requirements of a steady state.

m^{6}G-containing template is approximately the same as for G·C. However, the K_m^{app} for m^{6}G·C gives a limit of contamination of 1 G per 1500 m^{6}G, which cannot account for the low K_m of m^{6}G·T compared to G·T. In the case of the second template (Table 2), the opposite is true. The V_{max}^{rel} values are all lower than those of the normal pair, G·C, and the K_m^{app} values are diminished. These data are construed as strongly suggesting that contaminating G cannot be the basis for our results.

It is interesting that DNA polymerase favors insertion of dTMP over dCMP opposite m^{6}G even though m^{6}G·C base pairs appear to be more stable than m^{6}G·T, according to melting temperature measurements on synthetic polymers in aqueous solution (27). These results are in accord with studies on the templating properties of xanthine (28) and 2-aminopurine (29), in which relative nucleotide incorporation rates did not correlate with relative thermodynamic stabilities of mispairs involving analogues.

Our data appear to be consistent with Patel's ^{31}P-NMR data showing that an m^{6}G·T pair does not distort the phosphodiester backbone (10). Because hydrogen bonding alone does not account for the difference in m^{6}G·T pairing in the two templates, it appears that the stacking interaction of the 3′ base pair on its 5′ neighbor m^{6}G may be the important determinant.

Table 2. Kinetics of incorporation of dTTP or dCTP opposite m^{6}G 3′ to T in M13 DNA fragment m^{6}G-2



Pair	K_m^{app}, μM	V_{max}^{rel}	f
G·C (3)	0.11 ± 0.03	3.2 ± 0.9	1
G·T (3)	178 ± 46	0.8 ± 0.2	1.5×10^{-4}
m^{6}G·C (5)	21 ± 13	0.4 ± 0.1	6.6×10^{-4}
m^{6}G·T (4)	18 ± 7	0.4 ± 0.1	7.6×10^{-4}

See legend to Table 1.

The authors are grateful to Drs. Ashis Basu, Lynn Mendelman, Lawrence Loeb, and Sylvia Spengler for helpful discussions and criticisms. This work was supported by Grant CA 42736 (to B.S.) from the National Institutes of Health and was administered by Lawrence Berkeley Laboratory under Department of Energy Contract DE-AC0376SF00098 and by Grant GM 21422 (to M.F.G.) and Grant CA 43066 (to J.M.E.) from the National Institutes of Health.

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