N^4-Methoxydeoxycytidine triphosphate is in the imino tautomeric form and substitutes for deoxythymidine triphosphate in primed poly d[A-T] synthesis with E. coli DNA polymerase I

B.Singer¹, H.Fraenkel-Conrat², L.G.Abbott¹ and S.J.Spengler²

¹Laboratory of Chemical Biodynamics and Space Sciences Laboratory, University of California, Berkeley, CA 94730 and ²Department of Molecular Biology, University of California, Berkeley, CA 94720, USA

Received 11 April 1984; Revised and Accepted 15 May 1984

ABSTRACT

N^4-Methoxydeoxycytidine triphosphate (mo^dCTP) substitutes for dTTP in poly d[A-T] synthesis with E. coli DNA polymerase I (Pol I). In parallel experiments using as template-primer, poly d[G-C], no incorporation of [¹⁴C]mo^dC was detected. This indicates that this deoxy derivative acts as the imino tautomer, as previously found for the ribodervative. Nearest neighbor analysis of transcripts of poly d[A-T] containing mo^dC shows that the derivative substitutes for only one base. In replication, singlestranded mo^dC-containing polymers gave little misincorporation, including that of dATP which can hydrogen-bond to mo^dC in the imino form, if the methoxy group is anti to the N-3. It is therefore assumed that the methoxy group is constrained anti in a polymer such as d[A-T], but can be in the syn form in singlestranded polymers and not recognized by DNA polymerase. mo^dC destabilizes the poly d[A-T] helix, as indicated by a lowered and less cooperative melting. Steric factors such as adjacent base displacement were invoked for similar findings with the doublestranded r(U₆₁, mo^dC₃⁹)·r(A).

INTRODUCTION

Hydroxylamine and O-methylhydroxylamine (methoxyamine) have been useful reagents for studying the effect of tautomeric shift on mutation. Both form mono-substituted derivatives at the N of C (or dC), concommitant with addition to the doublebond¹. The N⁶ of A (or dA) reacts to only form the mono substituted derivative, but its reaction is much slower than that of the N⁴ of C¹. The known tautomeric equilibrium between amino and imino of N^4-hydroxy C has led to the use of precursor N^4-hydroxy CDP or N^4-hydroxy dCTP in site-directed mutagenesis²,³.

The methoxy analog, N^4-methoxy C (mo^dC), has been less studied but, in transcription, its presence has resulted in only ATP incorporation⁴. When poly (U₆₁, mo^dC₃⁹) is transcribed by DNA-dependent RNA polymerase with only ATP present, the resulting doublestranded polymer has a hydrogen-bonded structure, further showing that mo^dC substitutes for U as a result of being the imino tautomer⁵.

In this study we have prepared N^4-[¹⁴C]methoxydeoxycytidine triphos-
phate by a new method and use it as a substrate for polymerases. The effects of mo\textsuperscript{4}dC on the template properties of resulting polymers are reported.

MATERIALS AND METHODS

\textsuperscript{0-}[\textsuperscript{14}C]-methylhydroxylamine (methoxyamine) was obtained from New England Nuclear as a custom synthesis. Deoxynucleoside triphosphates were obtained unlabeled from Pharmacia P. L. and \textsuperscript{[3H]}-labeled from New England Nuclear at the highest specific activity available (10-30 Ci/mmol). \textsuperscript{[32P]} ribonucleoside triphosphates were from Amersham (600-3000 Ci/mM). Polynucleotides, deoxyoligomers, the Klenow fragment of Pol I, terminal deoxynucleotidyl transferase (TdT), and E. coli DNA-dependent RNA polymerase were from Pharmacia P. L. Avian myeloblastosis virus (AMV) reverse transcriptase was from Seikhaku America, Inc. Highly purified Pol I was a generous gift from Dr. L. A. Loeb.

Preparation of \textsuperscript{N-}[\textsuperscript{14}C]Methoxydeoxycytidine Triphosphate (mo\textsuperscript{4}dCTP)

25 mg dCTP (0.05 mmoles) were mixed with 0.15 ml 2.8 M pH 5.3 \textsuperscript{[14}C] methoxyamine HCl (0.42 mmoles, 0.12 mCi/mmol) and reacted for 18 h at 37°. To this was added 0.8 ml ethanol and 0.5 ml ether. The resulting oily precipitate was dissolved in 1 ml H\textsubscript{2}O. This crude product (yield 150 A\textsubscript{260} units, 6 x 10\textsuperscript{4} cpm/\lambda_{\text{max}}) was largely disubstituted (bis), as judged from a comparison with the spectrum with that of the ribocytidylic acid derivative. After evaporating to dryness with a N\textsubscript{2} stream, 0.5 ml 98% formic acid was added and the solution kept at 37° for 4 h, which almost quantitatively converted the bis to the \textsuperscript{N}-monosubstituted derivative. The product was chromatographed, descending, on Whatman 3 MM paper with isoprop alcohol: H\textsubscript{2}O:saturated ammonium sulfate (30:15:0.2) as solvent. mo\textsuperscript{4}dCTP moved near the solvent front and was clearly separated from dCTP, dCDP and the bis derivative. The spectra of the two compounds, bis and mono, are very different (Figure 1) and contamination of the mono with the bis is expressed by the ratio of the \lambda_{\text{max}}\textsubscript{220} to the \lambda_{\text{max}}\textsubscript{283}. The ratio obtained is 0.64, as contrasted to 0.75 for the previously reported analogous compound, \textsuperscript{N-}methoxycytidine-5'-phosphate. The final product, mo\textsuperscript{4}dCTP, in 0.1 N HCl had \lambda_{\text{max}}\textsubscript{220} at 220 nm and 283 nm, and \lambda_{\text{min}} at 219 nm and 245 nm. The integrity of the triphosphate was shown by chromatography on PEI-cellulose with 0.9 M L1Br which resolves mono-, di, and tri-phosphates. The specific activity was 2 x 10\textsuperscript{4} cpm/A\textsubscript{283} (pH 1).

Synthesis of Alternating Poly d[A-T,mo\textsuperscript{4}C]

Polymers were prepared using the same general method as previously
described\textsuperscript{7}. A typical reaction using Pol I contained (per ml): 50 mM Tris-HCl (pH 7.8), 2 mM MgCl\textsubscript{2}, 0.15 A\textsubscript{260} of poly d[A-T], 0.1 \mu mol dATP, 0.05 \mu mol dTTP, 0.05 mol \[^{14}C\]mo\textsuperscript{4}dCTP (2 x 10\textsuperscript{4} cpm/OD max) and 1.5 \mu g Pol I (30 units). After 75 min at 37\textdegree, the reaction was stopped and the polymer isolated\textsuperscript{7}. The yield in O.D. units was 4-fold the starting material, without corrections for losses on handling. The newly synthesized polymer contained, by radioactivity measurement, 5.2% mo\textsuperscript{4}dC which represents about 10\% of the pyrimidines. In another synthesis, using the Klenow fragment and stepwise procedure\textsuperscript{7} the net synthesis, after 8 h, was 7-fold without corrections, and 11.6% mo\textsuperscript{4}dC was incorporated. This represents 23\% of the pyrimidines.

**Attempted Synthesis of Poly d[G-C] Containing mo\textsuperscript{4}dC**

Poly d[G-C], in the presence of dGTP, dCTP, and mo\textsuperscript{4}dCTP (2:1:1), was used under the same experimental conditions, but on a larger scale than used to prepare poly d[A-T,mo\textsuperscript{4}dC]. Little decrease in absorbance occurred over a 6-hour period and on reisolation of the polymer, there was less than
two-fold synthesis. No $^{14}$C$m^4$C could be detected, although $1.5 \times 10^5$ cpms were in the reaction mixture.

**Synthesis of Single-stranded Deoxypolymers Containing $m^4$dC**

Terminal deoxynucleotidyI transferase (TdT) was used to prepare polymers with oligo(dT)$_7$ as primer. Reaction mixtures contained, per ml: 100 mM cacodylate buffer (pH 7.2), 10 mM MgCl$_2$, 1 mM CaCl$_2$, 2 mM 2-mercaptoethanol, 0.4 - 1.0 $\mu$mol of a mixture of dATP and $[^{14}$C]$m^4$dCTP (4:1 to 12:1 depending on the polymer desired), 0.1 A$_{260}$ primer and 1500 units TdT. The reaction was carried out in stopped cuvettes at 37° and monitored by the decrease in absorbancy. Maximum decrease in absorbancy (30%) and maximum yield required 72 h. The polymer was isolated by first separating it from dNTPs on cellulose thin layer sheets using as solvent Isopropanol:NH$_3$H$_2$O (55:25:10$^8$). The polymer which remains at the origin was scraped off and eluted with water. The eluate was further purified by passing it through a Biogel P-150 column (7 x 25 cm) using 10 mM pH 7.8 Tris as eluant. The yield, without corrections for handling, was 50-75%, which represents a polymer containing 300-400 nucleotides. The content of $m^4$dC was determined by counting an aliquot. Regardless of the input proportion of $m^4$dC, the final polymer contained about 65% of this input. Control polymers lacking $m^4$dC but containing unmodified triphosphates were prepared in a similar manner.

Attempts were made to use oligo (dC) or oligo (dA) as primers with $m^4$dCTP and dCTP but the polymer yield was very low. Radioactivity representing $[^{14}$C]$m^4$dC was incorporated but less than 15 nucleotides were added to the primer. These oligomers were too small to use as template.

**Nearest Neighbor Analysis of Transcripts**

Transcription and nearest neighbor analysis using $[^{32}$P]UTP or $[^{32}$P]ATP were according to Kusmierek and Singer$^9$. The incubation mixture contained 10 mM MgCl$_2$, 2 mM MnCl$_2$, 0.4 mM each of ATP, CTP, UTP, and GTP, with either UTP or ATP labeled, and E. coli DNA-dependent RNA polymerase.

**Assay of Fidelity in Replication**

Single-stranded deoxypolymers containing 4.5% or 8.5% $m^4$dC, 2% dC, 9% dT, or 4% dG were used for replication as follows. A 50 $\mu$l reaction mixture contained 0.01 A$_{260}$ template, mixed with 0.001 A$_{260}$ oligo (dT)$_{12-18}$, 0.1 mM each dTTP and one other dNTP, 50 mM Tris•HCl, pH 7.8, 2 mM MgCl$_2$ and 0.4 units Pol I. Duplicate experiments were performed with each labeled dNTP. Incubation was 15 min at 37°. 40 $\mu$l reaction mixture was spotted on DEAE disks and washed repeatedly with 7% Na$_2$HPO$_4$, then with H$_2$O, followed by ethanol. The disks were dried and then radioactivity counted using a toluene scintillant.
Blanks values were obtained from reaction mixtures which did not contain polymer. Misincorporation was considered significant only if the counts were at least double the blank. Reverse transcriptase from AMV was used under the same conditions except 20 units of enzyme were added and the reaction was for 30 min. One unit of reverse transcriptase is defined as incorporating 1 nanomole dTMP in 10 min, 35°, using an RNA template. One unit of Pol I incorporates 10 nanomoles of dTMP in 30 min 37° using an activated DNA template.

**Thermal Denaturation Measurement**

Measurements were made using Teflon-stoppered cuvettes in a dual-beam Varian-Cary 219 spectrophotometer equipped with jacketed sample and reference chambers. The temperature of the cell compartment was regulated by a Neslab Eva-Endocal programmed at 1°/2 min. Up to four samples can be compared simultaneously.

**RESULTS**

**mo4dCTP as a Substrate for Pol I**

[^14C]mo4dCTP and its bis derivative (N^4-methoxyamino-5,6-dihydro dCTP) were synthesized and characterized. The method of preparation of mo4dCTP utilized the fact that acid catalysis leads to reversal of the addition of the second methoxy group to the C5-C6 double bond. By means of 98% formic acid this result is achieved while avoiding any hydrolytic action on the phosphotriester or glycosidic bonds. The spectral properties and the availability of the resultant triphosphate for enzymatic incorporation into polynucleotides indicate a higher purity of this triphosphate than that of the ribo triphosphate, mo4CTP, previously reported. The yield was almost quantitative.

The monosubstituted triphosphate was a substrate for Pol I, was a competitor of dTTP, and was incorporated into poly [A-T]. Synthesis, with the two pyrimidines in a 1:1 ratio, was not decreased and mo4dCTP was incorporated with about 10% efficiency compared to dTTP. Using the Klenow fragment of Pol I, and increasing the amount of mo4dCTP relative to dTTP, the efficiency of incorporation of mo4dC could be increased to about 25%. In contrast, dCTP was not incorporated into poly d[A-T] at a level greater than 1/9000 bases synthesized. This is within the published values for the fidelity of Pol I11.

[^14C]mo4dCTP was not incorporated into poly d[G-C] with Pol I under the same conditions and, unexpectedly, its presence inhibited the synthesis of poly d[G-C].
Table 1. Nearest Neighbor Analysis of Transcripts of Poly (dA, mo^dC) and of Poly d(A-T, mo^dC) with [α^32P]UTP^d

<table>
<thead>
<tr>
<th>Polymer</th>
<th>C^32pU</th>
<th>A^32pU</th>
<th>G^32pU</th>
<th>U^32pU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-stranded^c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d[A-T]</td>
<td>0.21</td>
<td>904</td>
<td>2.7</td>
<td>4.4</td>
</tr>
<tr>
<td>d[A-T, mo^dC]</td>
<td>0.5</td>
<td>626</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Single-stranded^d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dA</td>
<td>0.24</td>
<td>0.48</td>
<td>0.55</td>
<td>433</td>
</tr>
<tr>
<td>dA, mo^dC</td>
<td>0.25</td>
<td>1.5</td>
<td>0.65</td>
<td>350</td>
</tr>
</tbody>
</table>

a Transcription conditions were according to Materials and Methods. DNA-dependent RNA polymerase, [α^32P]UTP, all four NTPs in equal molarity, 2 mM MnCl_2 and 10 mM Mg^2+ were used.

b The only significant misincorporation (> 2-fold that of the control polymer) resulting from mo^dC is underlined. 0.32% of the total transcription is Ap which represents 1 Ap/30 mo^dC.

c Prepared by Pol I synthesis. Poly d[A-T, mo^dC] contained 9.4% mo^dC.

d Prepared using terminal deoxynucleotidyl transferase with oligo dT, as primer. Poly (dA, mo^dC) contained 8.5% mo^dC.

Effect of mo^dC on Template Properties

DNA-dependent RNA polymerase was used to transcribe the mo^dC-containing polymers. Nearest neighbor analysis of the transcripts of alternating poly d[A-T] containing mo^dC, using [α^32P]UTP or ATP, showed that 99% of the ^32p was transferred to a single base, A or U respectively, (Tables 1, 2). These data indicate that the mo^dC is not randomly inserted, but is in alternating sequence. The only indication of altered fidelity in transcription of poly d[A-T, mo^dC] is the occasional incorporation of GTP (≈ 1/100 mo^dC) (Table 2). Single-stranded poly (dA, mo^dC), synthesized with an oligo (dT) primer, directed some incorporation of ATP (≈ 1/38 mo^dC) (Table 1).

The single-stranded polymers were also replicated using Pol I, but because of slippage commonly observed with stretches of dA hydrogen bonded to dT^{12}, the data are not easily interpreted. Although the presence of a few percent of dT in poly (dA) could easily be detected in this assay, mo^dC residues did not direct a proportional amount of dATP (Table 3). The high
Table 2. Nearest Neighbor Analyses of Transcripts of Poly d[A-T, mo^4C] with [32P]ATP^a

<table>
<thead>
<tr>
<th>Polymer</th>
<th>C^{32}pA (%)</th>
<th>A^{32}pA (%)</th>
<th>G^{32}pA (%)</th>
<th>U^{32}pA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d[A-T]</td>
<td>0.025 (&lt;0.01)</td>
<td>2.6 (0.35)</td>
<td>0.18 (0.038)</td>
<td>470 (&gt;99.5)</td>
</tr>
<tr>
<td>d[A-T, mo^4dC]</td>
<td>0.03 (&lt;0.01)</td>
<td>1.0 (0.25)</td>
<td>0.5 (0.12)</td>
<td>400 (&gt;99.5)</td>
</tr>
</tbody>
</table>

^a See Table 1 for description of methods and polymers.

^b Percent of total counts.

dATP incorporation with the control poly (dA) template made it impossible to ascertain whether any dATP incorporation could be attributed to mo^4dC. It was not possible to prepare polymers containing only dC and mo^4dC, which would have been advantageous in determining whether mo^4dC directed dA incorporation. No dCTP misincorporation was detected in poly (dA, mo^4dC) with either Pol I or AMV reverse transcriptase, which is more error-prone than Pol 1^13 (Table 3). There was a small but definite misincorporation of dGTP (1/30 - 1/100 mo^4dC) that was detectible with both DNA polymerases, as well as by nearest neighbor analysis of transcripts using DNA-dependent RNA polymerase (Tables 2, 3).

Effect of mo^4dC on Thermal Denaturation of Poly [A-T]

Poly d[A-T, mo^4C] destabilized the helix, as measured by T_m, to a measurable extent, but reannealing was complete. With 19% of the pyrimidines as mo^4dC, the T_m was decreased and the cooperativity was diminished (Table 4). Figure 2 illustrates the melting behavior of poly d[A-T] and poly d[A-T, mo^4dC].

DISCUSSION

Although Pol I has exonucleolytic activity which can excise mismatched bases during synthesis^13, mo^4dCTP is an acceptable substrate in poly d[A-T] synthesis. This is in accordance with our earlier finding that mo^4C forms hydrogen-bonded basepairs with A^5. This ability to basepair like T(U) can only occur if the derivative is the imino tautomer.

Both crystal^14 and nmr data^15,16 concur that mo^4C is in the imino form, but the exocyclic group prefers the rotational isomer which lies syn to the N-3 (Figure 3, top). Such a position would prevent basepairing. However, Engel and von Hippel^17 have shown that Pol I can rotate an exocyclic methyl group so that an adequate basepair can form (Figure 3, bottom). The ability
Table 3. Replication of Single-Stranded Deoxypolymers with Pol I and Reverse Transcriptase\(^a\)

<table>
<thead>
<tr>
<th>Polymer and Polymerase</th>
<th>dTTP + dATP</th>
<th>dTTP + dCTP</th>
<th>dTTP + dGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles</td>
<td>pmoles</td>
<td>pmoles</td>
</tr>
<tr>
<td>Pol I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dA</td>
<td>672</td>
<td>84</td>
<td>148</td>
</tr>
<tr>
<td>dA, mo(^d)dC (4.5%)</td>
<td>468</td>
<td>72</td>
<td>84</td>
</tr>
<tr>
<td>dA, dC (2%)</td>
<td>336</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>dA, dT (9%)</td>
<td>240</td>
<td>12 (1:2.4)(^d)</td>
<td></td>
</tr>
<tr>
<td>dA, dG (4%)</td>
<td>54</td>
<td>2.2</td>
<td>&lt;0.026</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dA</td>
<td>564</td>
<td>0.25</td>
<td>528</td>
</tr>
<tr>
<td>dA, mo(^d)dC</td>
<td>372</td>
<td>0.25</td>
<td>324</td>
</tr>
<tr>
<td>dA, dC</td>
<td>156</td>
<td>2.8</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>dA, dG</td>
<td>312</td>
<td>1.6</td>
<td>(1:8)(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Replication was as described in Materials and Methods. 1200 pmoles dT incorporation represents complete copying of the template. All values are calculated after subtracting the blanks obtained in the absence of polymer. Values less than double this number are indicated by a "less than" symbol. Each set of data represents an independent experiment.

The extent of replication was variable.

\(^b\) All polymers were synthesized using TdT and were initiated with oligo(dT)\(_{12-18}\) primers. The numbers in parentheses refer to the percent of each minor base in the polymer.

\(^c\) Pol I is highly purified E. coli DNA polymerase I. Reverse transcriptase (lot 83A011T) is the DNA polymerase from AMV.

\(^d\) The numbers in parentheses refer to the incorporation of dA or dC or dG relative to the amount of the complement in the template. For example, with 9% T in a template and 3.7% A in the replicated portion, the ratio (or efficiency) is 1 dA/2.4 dT after subtracting the control, dA lacking dT.

Table 4. Thermal Denaturation of poly d[A-T] Containing mo\(^d\)dC

<table>
<thead>
<tr>
<th>Polymer</th>
<th>15 mM NaCl/1.5 mM Na citrate, pH 7</th>
<th>1 M NaCl(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T(_m), °C</td>
<td>H, °C</td>
</tr>
<tr>
<td>Poly d[A-T, mo(^d)dC](^c)</td>
<td>42.4</td>
<td>39.4</td>
</tr>
<tr>
<td>Poly d[A-T]</td>
<td>46.1</td>
<td>41.9</td>
</tr>
</tbody>
</table>

\(^a\) also contains 15 mM NaCl/1.5 mM Na citrate, pH 7.

\(^b\) Width of transition (°C) from 25-75% of total hyperchromicity.

\(^c\) Contains 9.4% mo\(^d\)dC.
Fig. 2. Thermal melting profiles in 1 M NaCl containing 0.1 x SSC. Poly d[A-T, mo^4C] contains 11.5% mo^4dC, which is 23% of the pyrimidines.

of a polymerase to rotate an exocyclic group may vary with the specific group and may be energetically favorable in polymerization.

Once the modified nucleotide is incorporated into a double-stranded structure it likely to continue to be held anti so that basepairing is maintained. This would, however, destabilize the polymer, which we find for the alternating polymer, poly d[A-T, mo^4C], (Figure 2, Table 4).

The lack of significant A or dA incorporation in transcription and replication of single-stranded dA polymers containing mo^4dC is not, we

Fig. 3. (Top) The anti and syn conformations of the imino form of mo^4C. (Bottom) Watson-Crick type basepairing between dAdo and mo^4dC in the anti imino form. The drawing is adapted from Kierdaszuk et al.17.
believe, an accurate reflection of the nucleotide's ability to pair with A. This has been demonstrated with the same enzymes, Pol I and DNA-dependent RNA polymerase, in the experiments with the alternating polymer (Tables 1,2) and in earlier work from this laboratory. Thus, it is likely that failure to observe dA incorporation using poly(dA, mo4dC)templates is a consequence of some aspect of the structure of the single-stranded polymer. There is significant stacking in poly (dA), even greater than observed in poly(rA). Therefore, mo4dC could be excluded by these interactions and not read by the polymerase. Indeed, inclusion of mo4dC might disrupt not only the specific stacking of two residues, but also delay the renucleation of stacking by distorting the alignment. It is well established that the pyrimidines do not stack as well as the purines, which could account for the observation that in copolymers with C appropriate mo4C•A pairing occurred. Therefore, the expression of mo4C•A pairing may be sequence dependent.

A secondary factor which addresses the very low, but consistent, G incorporation directed by these polymers is the possibility that the small proportion of mo4C in the anti amino form can be read. Here again, the flexibility about the N-O bond would permit discrete insertion of the nucleotide in the helix, as if it were C. It appears unlikely that even the presumed minor proportion of mo4dCTP in the amino form can substitute for the unmodified triphosphate. In fact, the presence of mo4dCTP greatly inhibits Pol I synthesis of poly d[G-C]. A similar inhibition has been observed when m6dGTP is substituted for dGTP in poly d[G-C] synthesis.

It was suggested by Kierdaszuk and Shugar, from model studies in chloroform, that the presence of a methyl group on the C-5 would be even more energetically unfavorable for the methoxy group to rotate anti. However, in unpublished experiments from this laboratory, we find that 5-methyl-N4-methoxy CTP, incorporated into ribopolymers, directs the incorporation of rA only slightly less frequently than does mo4C. Thus, we conclude that the methoxy group of mo4C which is predominantly imino can be rotated by polymerases in solution.

ACKNOWLEDGEMENT

This work was supported by Grant CA 12316 from the National Cancer Institute, National Institutes of Health, Bethesda, MD. S.J.S. was the recipient of an American Cancer Society Senior Fellowship.
REFERENCES