Base Dynamics of Nitroxide-Labeled Thymidine Analogues Incorporated into (dA-dT)$_n$ by DNA Polymerase I from *Escherichia coli*[†]

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ABSTRACT: Nitroxide-labeled thymidine substrates (dL) for *Escherichia coli* DNA polymerase I (pol I) were used to synthesize spin-labeled alternating double-stranded copolymers with (dA-dT)$_n$ as a template. All dL substrates use an alkane or alkene tether substituted into the 5-position of the pyrimidine ring to link a five- or six-membered ring nitroxide to the pyrimidine base. The kinetics of dL incorporation show some tether dependence with respect to tether length and tether geometry. The electron spin resonance (ESR) spectra of (dA-dT,dL)$_n$ duplexes directly formed by polymerization with pol I are compared with the ESR spectra of (dA)$_n$(dT,dL)$_n$ duplexes, which are obtained after annealing of nitroxide-labeled single strands with complementary unlabeled single strands. The ESR spectra indicate that nitroxide-labeled analogues with tethers short enough to let the nitroxide ring reside in the major groove are excellent reporter groups for monitoring hybridization. A small difference between the ESR line shapes of the alternating duplexes (dA-dT,dL)$_n$ and the homopolymer duplexes (dA)$_n$(dT,dL)$_n$ containing the same dL is detectable, suggesting the presence of subtle differences in the base dynamics between both systems. Computer simulation of the ESR spectra of the (dA-dT,dL)$_n$ duplexes was successful with the same motional model reported earlier [Kao, S.-C., & Bobst, A. M. (1985) *Biochemistry 24*, 5465–5469]. The thymidine motion arising from tilting and torsion of base pairs and base twisting in (dA-dT)$_n$ is similar to that in (dA)$_n$(dT)$_n$ and is of the order of 4 ns.

The use of spin-labeling to study some characteristics of nucleic acids such as their base dynamics, their interactions with complementary nucleic acids, and their interactions with nucleic acid binding proteins and drugs has been well documented in recent years in this laboratory (Kao et al., 1983, 1985; Bobst et al., 1984a; Ireland et al., 1986). Originally, spin-labels were introduced into a variety of preexisting nucleic acid lattices by chemical modification reactions [for reviews, see Dugas (1977), Bobst (1979), and Kamzolova and Postnikova (1981)] or with spin-labeled intercalators (Mil et al., 1980; Robinson et al., 1980; Hurley et al., 1982). For the most recent review on spin-labeled nucleic acids, also see Robinson et al. (1985). To achieve site-specific labeling with respect to the nucleic acid base, spin-labeled ribonucleic and deoxyribonucleic acids were prepared with the aid of template-independent enzymes using as substrates unmodified nucleotides together with nucleotide analogue sites specifically spin-labeled at the 4- or 5-position of a pyrimidine base. The copolymerization reactions were performed with polynucleotide phosphorylase and ribonucleotide diphosphates (Bobst & Torrence, 1978) to make RNA, and with terminal deoxynucleotidyltransferase and deoxyribonucleic triphosphates (Toppin et al., 1983) to make DNA. These enzymatic procedures yielded spin-labeled single-stranded nucleic acids which then were annealed with complementary unlabeled single strands to form RNA and DNA duplexes.

The electron spin resonance (ESR)[1] spectra of these duplexes provided good experimental evidence for base-dependent rapid motions on a nanosecond time scale in nucleic acids. Substantial changes in the nucleic acid geometry are attributed to motions in the time range from 1 to 100 ns according to the Barkley–Zimm calculations (Barkley & Zimm, 1979). A variety of techniques have recently been used to study these dynamic motions in (dA)$_n$(dT)$_n$ and (dA-dT)$_n$ (Assat-Munt et al., 1984; Behling & Kearns, 1986; Wartell & Harrell, 1986).

We now report our findings on the incorporation of the novel thymidine triphosphate analogues ppp-DUAP, ppp-DUAT, ppp-DUPAT, and ppp-DUAVAP shown in Figure 1 with the template-dependent DNA polymerase pol I from *Escherichia coli* using (dA-dT)$_n$ as a template. Some preliminary observations reported with ppp-DUAT (Toppin et al., 1986) suggested that analogues of this type may serve as efficient substrates for pol I. Indeed, we found that they can all be incorporated by pol I to give double-stranded (dA-dT,dL)$_n$ with an extent of incorporation varying with the nature of the tether. ppp-DUPAT was the poorest substrate while ppp-DUAVAP competed almost as effectively as TTP for pol I with the (dA-dT)$_n$ template. Of particular interest is a comparison of the ESR line shapes of these spin-labeled duplexes with the ESR line shapes reported earlier for duplexes obtained upon annealing of a spin-labeled single strand with its comple-

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[1] Abbreviations: structures of the abbreviated deoxyuridine analogues (dL) DUAT, DUPAT, DUAP, and DUAVAP are given in Figure 1; p-dL and ppp-dL, mono- and triphosphates of the dL, respectively; TTP, thymidine 5'-triphosphate; dATP, 2'-deoxyadenosine 5'-triphosphate; DNA, deoxyribonucleic acid; (dT,dL)$_n$, single-stranded homopolymer of thymidine in which some thymidine residues are randomly replaced by DUPAT; (dA)$_n$(dT)$_n$, duplex formed by annealing of (dA)$_n$ with (dT)$_n$; (dA)$_n$(dT,dL)$_n$, duplex formed by annealing of (dA)$_n$ with (dT,dL)$_n$; (dA-dT)$_n$, alternating copolymer of deoxyadenosine and thymidine; (dA-dL)$_n$, alternating copolymer of deoxyadenosine and any spin-labeled thymidine analogue (dL); (dA-dT,dL)$_n$, any alternating copolymer of deoxyadenosine and thymidine in which the spin-labeled thymidine analogue dL randomly replaces some of the thymidine bases; pol I, DNA polymerase I from *E. coli*; ESR, electron spin resonance; $\tau_\parallel$ and $\tau_\perp$, correlation times for rotations about and perpendicular to the principal axis of diffusion, respectively; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; EDTA, ethylenediaminetetraacetic acid.
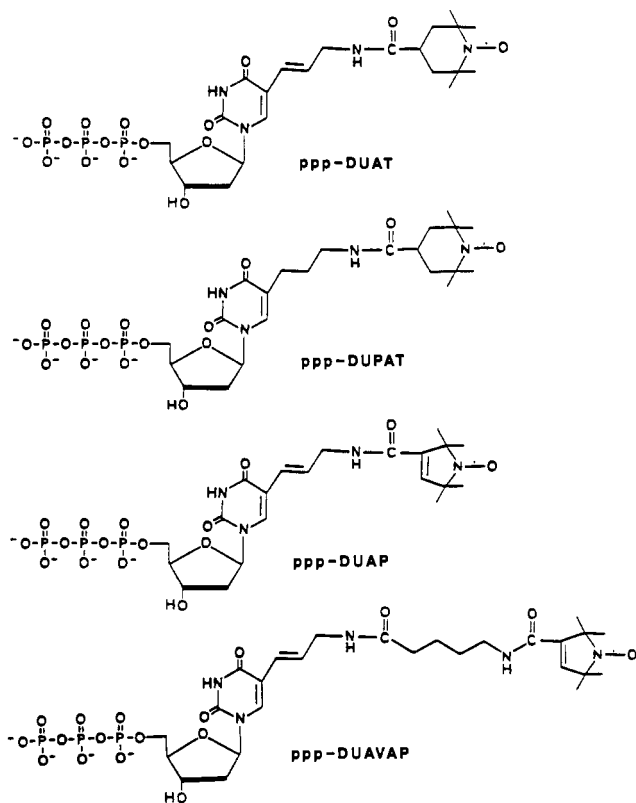
FIGURE 1: Chemical structures of the nitroxide spin-labeled thymidine analogues ppp-DUAT, ppp-DUPAT, ppp-DUAP, and ppp-DUAVAP.

mentary unlabeled strand. It was also possible to detect strong ESR line-shape broadening attributable to Heisenberg spin exchange in double-stranded DNA with high DUAVAP label incorporation.

MATERIALS AND METHODS

Nonradioactive triphosphates, $(dA-dT)_n$, terminal deoxynucleotidyltransferase, and *E. coli* DNA polymerase I were purchased from *Pharmacia*. [8-^{3}H]Deoxyadenosine 5'-triphosphate was purchased from ICN. All ESR spectra were recorded with a Varian E-104 Century Series spectrometer interfaced with an Apple II plus microcomputer (Ireland et al., 1983). The microwave power was 10 mW, and the modulation amplitude was 1 G for the spin-labeled building blocks and 1.6 G for the spin-labeled polymers. The ESR measurements were determined with an E 248-1 Varian flat quartz cell in an E-238 cavity. Simulations of ESR spectra were carried out with the same motional model as reported earlier (Kao et al., 1983; Kao & Bobst, 1985) with the following ESR parameters: $\tau_\parallel$ = 0.06 ns, $\tau_\perp$ = 1.2 ns, T_2^{-1} = 1.2 G, tilt angle = 55° for the single-stranded system (dT, DUPAT)$_n$; $\tau_\parallel$ = 0.2 ns, $\tau_\perp$ = 4 ns, T_2^{-1} = 0.8 G, tilt angle = 50° for the double-stranded systems $(dA)_n(dT,DUPAT)_n$ and $(dA-dT,DUPAT)_n$. The same A and g tensors were used for all systems and consisted of A_{xx} = 7.35 G, A_{yy} = 7.35 G, A_{zz} = 35.6 G, g_{xx} = 2.0088, g_{yy} = 2.0059, and g_{zz} = 2.0026. The line-broadening term T_2^{-1} (Freed, 1976), which reflects broadening by unresolved proton splittings and other interactions independent of motion, was obtained as described earlier (Kao & Bobst, 1985).

The spin-labeled thymidine triphosphate analogues ppp-DUAT, ppp-DUPAT, ppp-DUAP, and ppp-DUAVAP, shown in Figure 1, were used as their ammonium salts. The synthesis of ppp-DUAT is given in Toppin et al. (1985). The synthesis of ppp-DUPAT, ppp-DUAP, and ppp-DUAVAP is similar to that of ppp-DUAT and will be reported elsewhere. Figure 2
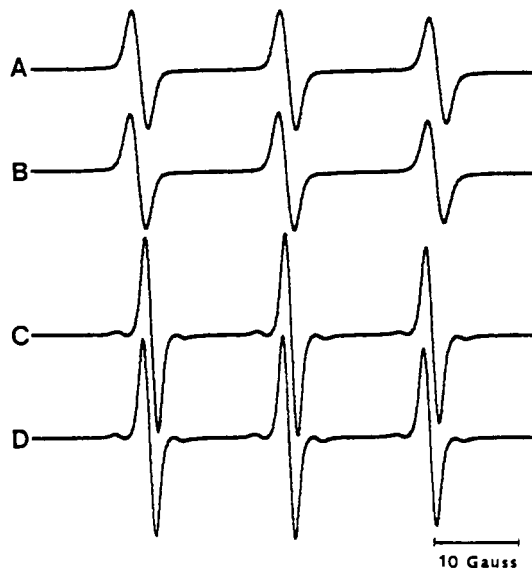

FIGURE 2: ESR spectra of 3.2 × 10^{-5} M solutions of (A) ppp-DUAT, (B) ppp-DUPAT, (C) ppp-DUAP, and (D) ppp-DUAVAP in 0.01 M NaCl/0.01 M sodium cacodylate buffer (pH 7). ESR gain is the same for all spectra.

shows the ESR spectra of the spin-labeled nucleotide triphosphates.

Preparation of $(dT,DUPAT)_n$ and $(dA)_n(dT,DUPAT)_n$. The single-stranded polymer $(dT,DUPAT)_n$ was prepared by copolymerization of TTP and ppp-DUPAT with terminal deoxynucleotidyltransferase as described in Toppin et al. (1983). The homopolymer duplex $(dA)_n(dT,DUPAT)_n$ was formed by annealing equimolar quantities of $(dA)_n$ with $(dT,DUPAT)_n$.

Preparation of $(dA-dL)_n$. By a modification of the method of Schachman et al. (1960), polymerization reactions (120 μL) were carried out in polypropylene tubes under the following conditions: 66 mM KH$_2$PO$_4$, pH 7.5, 0.1 mM *dl*-dithiothreitol, and 6.2 mM MgCl$_2$ with 60 nmol of [^{3}H]dATP (60 Ci/mol), 60 nmol of TTP or spin-labeled TTP analogue, and 7.6 nmol (in nucleotide units) of $(dA-dT)_n$ template. This mixture was preincubated at 37 °C for 10 min prior to addition of 3 units of *E. coli* polymerase I. Samples (10 μL) were withdrawn at appropriate time intervals and spotted onto Whatman DE81 filters. The filters were washed 6 times with 0.2 M Na$_2$HPO$_4$, 3 times with water, and 2 times with 95% ethanol, dried, and counted in a toluene-based scintillation fluid (PPO/POPOP).

Preparation of $(dA-dT,dL)_n$. Various amounts of ppp-DUAT, ppp-DUAP, ppp-DUPAT, or ppp-DUAVAP were polymerized with constant amounts of TTP and dATP with $(dA-dT)_n$ as a template-primer. Reactions (1.22 mL) were carried out in polystyrene tubes under the buffer conditions listed above with 0.6 μmol of dATP, 0.3 μmol of TTP, various amounts of labeled thymidine triphosphate analogue from 0.075 up to 0.3 μmol, and 0.5 absorbance 262-nm unit (75 nmol) of $(dA-dT)_n$. This mixture was preincubated at 37 °C for 10 min before addition of 15–30 units of pol I. The progress of the reaction was followed by periodically transferring a sample of the reaction to a 2-mm path-length cell and determining the absorbance at 262 nm. When the absorbance decrease reached a minimum, the reaction was terminated with the addition of 0.4 mL of 3.0 M NaCl/0.045 M Na$_2$EDTA. Reactions were generally run from 45 to 100 min. The time before the 3'-5'-exonuclease activity became dominant and the extent of hypochromic decrease varied between enzyme lots. The reactions were deproteinized by ex-
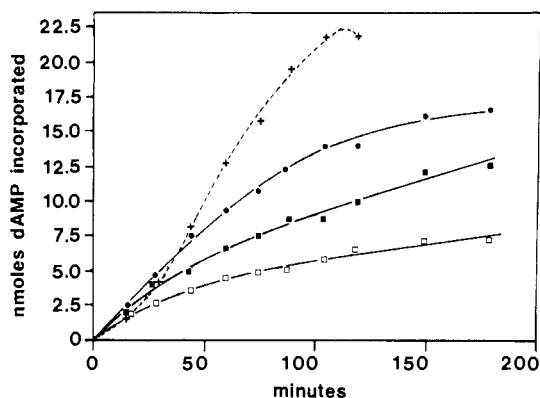
FIGURE 3: Incorporation of [^{3}H]dATP into (dA-dL)$_n$ as a function of time. (+) (dA-dT)$_n$ control reaction; (●) (dA-DUAVAP)$_n$; (■) (dA-DUAP)$_n$; (□) (dA-DUAT)$_n$.

FIGURE 4: Hypochromic decrease in absorbance at 262 nm observed during the time course of polymerization into (dA-dT,dL)$_n$. Reactions contain 0.6 μmol of dATP and (+) 0.6 μmol of TTP to make (dA-dT)$_n$, (●) 0.3 μmol of TTP and 0.3 μmol of ppp-DUAVAP to make (dA-dT,DUAVAP)$_n$, (■) 0.3 μmol of TTP and 0.3 μmol of ppp-DUAP to make (dA-dT,DUAP)$_n$, (□) 0.3 μmol of TTP and 0.3 μmol of ppp-DUAT to make (dA-dT,DUAT)$_n$, and (O) μmol of TTP and 0.3 μmol of ppp-DUPAT to make (dA-dT,DUPAT)$_n$.

traction with chloroform/isoamyl alcohol (5:2 v/v). The polymerized material was separated from the building blocks by exclusion chromatography on a Sephacryl S-200 column eluted with 0.04 M ammonium bicarbonate, pH 7.5. The samples were then frozen, lyophilized, and stored at -20 °C until needed. The typical yield of a polymerization reaction is from 1.5 to 3.0 absorbance 262-nm units (225–450 nmol).

Characterization of Polymers. Aliquots of the spin-labeled nucleic acids are digested with S1 nuclease from *Aspergillus oryzae*, and the ESR signal amplitudes of the digests are compared with the ESR signal amplitudes of known amounts of p-DUAT or p-DUAP which were dissolved in the digestion buffer and had been subjected to the digestion conditions. The digestion is carried out in 50 mM NaCl, 30 mM sodium acetate, and 1 mM ZnSO$_4$ buffer, pH 4.6, containing 5% v/v glycerol with 500–700 units of S1 nuclease. The percentage of probe incorporation is calculated as probe concentration over polymer concentration times 100, and the reported corrected incorporation value is calculated by subtracting the amount of (dA-dT)$_n$ which served as the polymerization primer. All enzymatically spin-labeled nucleic acids were examined for their size by gel electrophoresis. The homopolymers obtained with terminal deoxynucleotidyltransferase consisted of 300–600 nucleotides, whereas the alternating copolymer duplexes synthesized with pol I contained a minimum of 1000 nucleotides.

RESULTS

The polymerization reactions carried out in this study can be divided into two distinct groups. In the first one, there are only spin-labeled substrates in place of TTP in the reaction mixture, whereas in the second group the labeled substrates have to compete with TTP for incorporation by pol I.

When the spin-labeled thymidine triphosphate analogue (dLTP) fully substitutes for TTP as shown in Figure 3, the determination of incorporated [^{3}H]dAMP is a measure of the incorporation rate of the dLTP, which reflects the acceptability of these analogues as substrates for pol I. As is apparent from Figure 3, ppp-DUAVAP, ppp-DUAP, and ppp-DUAT each display a distinct incorporation rate. ppp-DUAVAP is definitely the best substrate, while ppp-DUPAT showed no noticeable incorporation under these conditions.

In Figure 4, the dLTP and TTP are present at a ratio of 1:1 in the polymerization reaction, which is monitored by following the hypochromic decrease of UV absorbance at 262 nm. Typically, the absorbance of a reaction mixture is seen to decrease as monomeric nucleotide triphosphates are incorporated into the duplex lattice. This hypochromic decrease in absorbance continues as long as polymerization is in
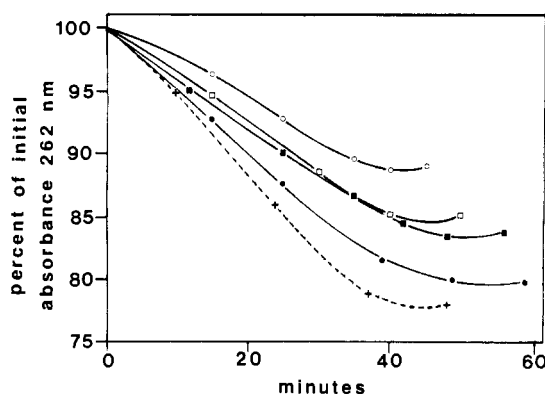
progress. At some point, the polymerase activity of the enzyme levels off, and the 3'-5'-exonuclease activity becomes dominant (Kornberg, 1965). As a result, the absorbance of the reaction mixture reaches a minimum and then begins to increase.

It was noticed that replacing some or all of the TTP with labeled analogue reduces the rate of this exonuclease activity. The degree of inhibition appears to be related to the percentage of labeled nucleotides in the lattice. In reactions in which a labeled analogue fully replaced TTP, polymerization went on without apparent exonuclease activity for several hours (see Figure 3). Actually, after 8 h of polymerization, the total synthesis of (dA-DUAP)$_n$ as determined by [^{3}H]dAMP incorporation was nearly double the amount obtained in a control experiment with no spin-labeled substrate (data not shown).

The rates of hypochromic decrease determined during the synthesis of (dA-dT,dL)$_n$ (Figure 4) follow a similar rank of order as the rates measured by [^{3}H]dAMP incorporation to make (dA-dL)$_n$ (Figure 3). In addition, the total percent of absorbance change shown in Figure 4 before the exonuclease activity becomes apparent is in agreement with the incorporation data of Figure 3. Namely, as can be seen in Figure 4 with a polymerization reaction containing TTP and ppp-DU-PAT in a 1:1 ratio, the extent of the reaction reflects what may be expected from the TTP component alone with little or no contribution from ppp-DUPAT. Taking this as a reference, the data shown for ppp-DUAT, ppp-DUAP, and ppp-DUAVAP show that these compounds contribute to the polymerization with a relative efficiency comparable to that seen in Figure 3. Similar observations were made by Sagi et al. (1977), who studied the incorporation rate of various 5-alkyl-dUTP compounds into a (dA-dT)$_n$ lattice by pol I. Also, since these polymerization reactions result in severalfold replication of the original (dA-dT)$_n$ template (see Materials and Methods), it is likely that the newly formed (dA-dT,dL)$_n$ can serve as a template. This assumption is supported by the findings of Sagi and Otvos (1979), who found various (dA-5-alkyl-dU)$_n$ copolymers to be effective template-primers for the polymerization of (dA-dT)$_n$ by pol I.

The relationship between the ESR signal amplitude and the percentage of labeled bases present in a DNA polymer depends on various factors. These include the type of spin-labels used (five- or six-membered ring nitroxide), the properties of the tether linking the probe to the nucleotide base, the total amount of spins present in the lattice (Heisenberg spin exchange), and the conformational state of the nucleic acid. For these reasons,
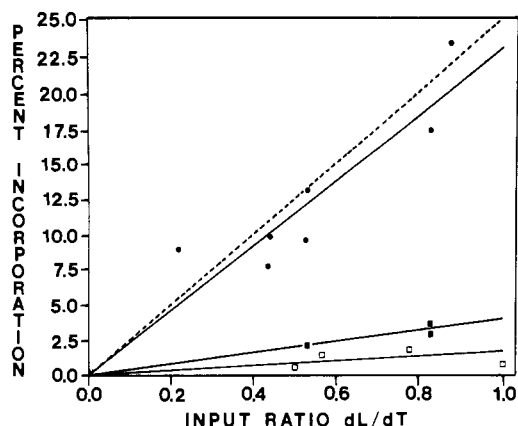
FIGURE 5: Percent spin-label content of (●) (dA-dT,DUAVAP)$_m$ (■) (dA-dT,DUAP)$_n$, and (□) (dA-dT,DUAT)$_n$ as a function of input ratio of labeled thymidine triphosphate analogue/thymidine triphosphate in the polymerization reaction. No least-squares line is shown for (dA-dT,DUPAT)$_n$ as this line would be indistinguishable from the abscissa (see text for incorporation levels of this compound). (---) represents calculated data of ideal thymidine analogue incorporation at a given input ratio.

Table I: Parameters of Free and Incorporated Spin-Labeled Nucleotides[a]

	dL/dN[b] ratio	h_{+1}/h_0 (±0.01)	h_{-1}/h_0 (±0.02)	$h_{0,p-p}$ (G)	a (G)
building blocks					
ppp-DUAT	N/A[c]	0.98	0.89	2.0	16.6
ppp-DUPAT	N/A	0.97	0.87	2.0	16.6
ppp-DUAP	N/A	0.96	0.87	1.5	15.6
ppp-DUAVAP	N/A	0.97	0.87	1.5	15.6
single strands					
(dT,DUAT)$_n$	0.02	0.98	0.60	2.4	N/A
(dT,DUPAT)$_n$	0.02	0.97	0.60	2.4	N/A
double strands					
(dA)$_n$(dT,DUAT)$_n$	0.01	0.73	0.21	2.5	N/A
(dA-dT,DUAT)$_n$	0.012	0.75	0.24	2.5	N/A
(dA)$_n$(dT,DUPAT)$_n$	0.01	0.97	0.25	2.5	N/A
(dA-dT,DUPAT)$_n$	0.002	0.90	0.29	2.5	N/A
(dA-dT,DUAP)$_n$	0.048	0.51	0.24	2.1	N/A
(dA-dT,DUAVAP)$_n$	0.089	0.90	0.59	2.0	N/A

[a] Solvent system: 0.01 M NaCl/0.01 M sodium cacodylate (pH 7). [b] Spin-labeled analogues to total nucleotides. [c] N/A, not applicable.

it is necessary to digest the spin-labeled nucleic acid lattices and to compare the ESR spectra of the digests with standard solutions (see Materials and Methods) in order to determine the extent of spin incorporation. Figure 5 gives the percentage of spin incorporation as a function of the ratio of labeled analogue versus TTP under conditions that keep the total amount of TTP and dATP constant. This approach will allow a determination of the ability of the analogues to compete with TTP for incorporation into a (dA-dT)$_n$ lattice by pol I. The dashed line in Figure 5 represents an ideal TTP analogue, which at an input ratio of TTP analogue/TTP of 1.0 should be incorporated to an extent of 50%. Since the lattice itself consists of half thymidine and half adenosine, the incorporation of the analogue with respect to the newly formed lattice should be 25% at an input ratio of 1.0.

As can be seen in Figure 5, ppp-DUAVAP competes very well with TTP for incorporation by pol I. ppp-DUAP and ppp-DUAT compete to a lesser degree but are still incorporated into the lattice to an extent that they can be readily detected by ESR. ppp-DUPAT is the least competitive analogue with pol I and was found to give an incorporation percentage of no greater than 0.2%.

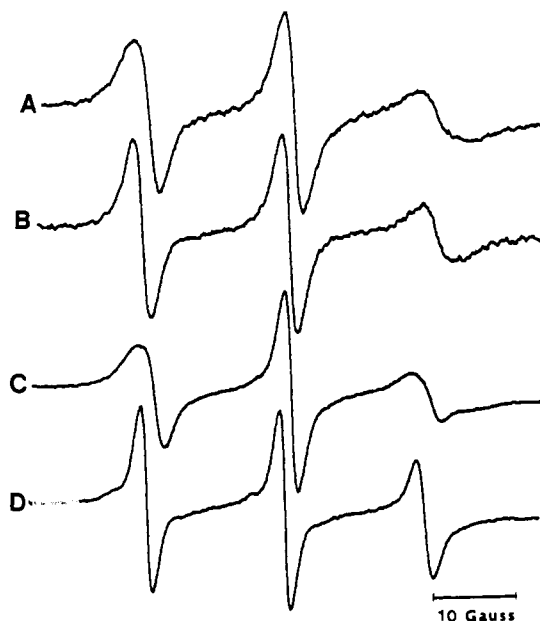The ESR spectra of the polymers obtained with pol I and the four novel spin-labeled substrates are shown in Figure 6.

FIGURE 6: ESR spectra of (A) 1.8 × 10^{-4} M (dA-dT,DUAT)$_n$ [dL/(dL + dT + dA) = 0.012], (B) 5.8 × 10^{-4} M (dA-dT,DUPAT)$_n$ [dL/(dL + dT + dA) = 0.002], (C) 2.3 × 10^{-4} M (dA-dT,DUAP)$_n$ [dL/(dL + dT + dA) = 0.048], and (D) 3.2 × 10^{-5} M (dA-dT,DUAVAP)$_n$ [dL/(dL + dT + dA) = 0.089] in 0.01 M NaCl/0.01 M sodium cacodylate (pH 7). The intensity of the spectra have been normalized to set the amplitude H_0 equal in all spectra.

Table I summarizes some of their spectral characteristics as well as those of (dT,DUPAT)$_n$ and (dA)$_n$(dT,DUPAT)$_n$ obtained with terminal deoxynucleotidyltransferase. In addition, we include for comparative purposes some line-shape parameters of (dT,DUAT)$_n$ and (dA)$_n$(dT,DUAT)$_n$ whose simulation parameters were reported earlier (Kao & Bobst, 1985). Table I also contains some parameters of the spectra shown in Figure 2.

Spectrum A in Figure 6 is that of (dA-dT,DUAT)$_n$ and is very similar to the ESR spectrum of (dA)$_n$(dT,DUAT)$_n$ shown in Kao and Bobst (1985). There is a subtle difference between the two systems with regard to the h_+/h_0 ratios which is beyond the error limit (Table I). Also, a close examination of the h_+ first-derivative signal reveals some asymmetry with the spin-labeled alternating copolymer which was absent in (dA)$_n$(dT,DUAT)$_n$. It should be noticed, however, that the ESR line-shape differences between both systems are very subtle and the experimental spectra of both systems can be computer simulated with a good fit using the same input parameters. These parameters were published earlier for (dA)$_n$(dT,DUAT)$_n$ (Kao & Bobst, 1985). A reduction of the double bond in the tether of DUAT gives rise to DUPAT. The alternating copolymer containing DUPAT, (dA-dT,DUPAT)$_n$, gives a characteristic spectrum shown in Figure 6B. From a comparison of spectra A and B, and the parameters given in Table I, it is clear that the loss of the double bond mainly affects the amplitude of the h_+ signal. A possible explanation for this increase is obtained from computer-simulated spectra and is given below with Figure 7.

The remaining two ESR spectra of Figure 6 show the results obtained with spin-labeled nucleic acids containing nucleotides labeled with the five-membered nitroxide ring. With DUAP in (dA-dT,DUAP)$_n$, which has a tether of the same length as DUAT, one obtains spectrum C. Its line shape is characteristic of a spin-labeled tether short enough to force the five-membered nitroxide ring to remain in the physically restrictive area of the major groove. No attempt has been made so far to computer simulate the spectra of five-membered ring ni-
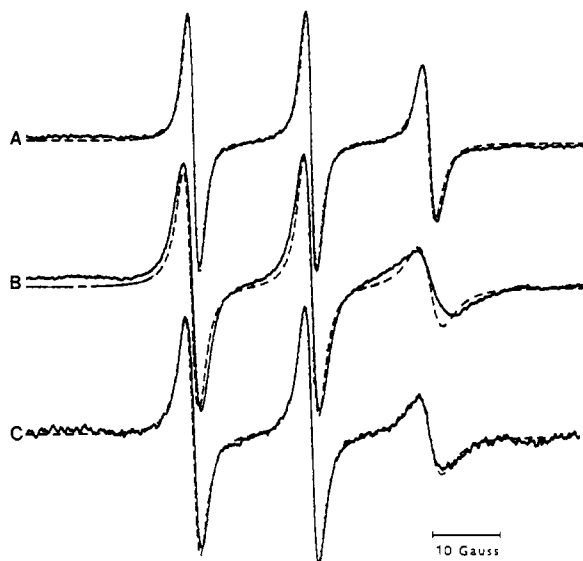
FIGURE 7: Experimental (—) and computer-simulated (---) ESR spectra of (A) 0.7×10^{-4} M $(dT,DUPAT)_n$ $[dL/(dL + dT) = 0.02]$, (B) 1.4×10^{-4} M $(dA)_n(dT,DUPAT)_n$ $[dL/(dL + dT + dA) = 0.01]$, and (C) 5.8×10^{-4} M $(dA\text{-}dT,DUPAT)_n$ $[dL/(dL + dT + dA) = 0.002]$. The experimental spectra were recorded in 0.01 M NaCl/0.01 M sodium cacodylate (pH 7). The computer-simulated spectra were calculated as described under Materials and Methods.

troxide-labeled nucleic acids, since such simulations will necessitate the determination of g and A tensors which differ from six-membered nitroxide-labeled systems. With $(dA\text{-}dT,DUAVAP)_n$, a five-membered nitroxide-labeled nucleic acid, one observes spectrum D. This spectrum is characteristic of a nitroxide label residing outside of the major groove as was shown earlier with the long leg label DUMBT in $(dA)_n$ $(dT,DUMBT)_n$ (Bobst et al., 1984b). Thus, five-membered ring nitroxide labels attached to appropriate tethers can also serve as a dipstick of the major groove of nucleic acids.

A careful examination of the line shapes of the six-membered ring nitroxide-labeled nucleic acids is given with Figure 7. In particular, the effect of the double bond in the tether on the ESR spectra is analyzed. Spectrum A is obtained with $(dT,DUPAT)_n$, and the parameters for the simulated spectrum are the same as those reported earlier for $(dT,DUAT)_n$ (Kao & Bobst, 1985). Thus, the presence or absence of a double bond in the tether seems to have little effect on $\tau_\perp$, which in the motional model (Kao et al., 1983) is believed to reflect motions about the principal axis of diffusion z' and was defined along the tether pyrimidine linkage. A similar observation was made earlier when essentially the same spectra were obtained with the single strands $(DUAT,dT)_n$ and $(DUMPT,dT)_n$ (Kao & Bobst, 1985). The tethers in DUAT and DUMPT are similar in length, but the latter, like DUPAT, contains no double bond in the tether.

For the double-stranded systems $(dA)_n(dT,DUPAT)_n$ and $(dA\text{-}dT,DUPAT)_n$, one gets spectra B and C, respectively, shown in Figure 7. Both systems exhibit similar line shapes and on a first approximation can be computer simulated with the same input parameters, which correspond to the values published earlier for $(dA)_n(dT,DUAT)_n$ and $(dA)_n(dT, DUMPT)_n$ (Kao & Bobst, 1985) with the exception of the tilt angle. $\tau_\parallel$ was shown to be of the order of 0.2 ns for the duplexes containing DUMPT and DUAT with a tilt angle of $40°$, which is the angle between the diffusion and magnetic axes. For its exact definition, see Figure 2 in Kao et al. (1983) or Figure 3 in Kao and Bobst (1985). Thus, the major effect of reducing the double bond in the tether of a DUAT incorporated into a DNA duplex is an increase of the tilt angle from

FIGURE 8: ESR spectra of 3.2×10^{-4} M $(dA\text{-}dT,DUAVAP)_n$ $[dL/(dL + dT + dA) = 0.089]$ (—) and 6.8×10^{-5} M $(dA\text{-}dT, DUAVAp)_n$ $[dL/(dL + dT + dA) = 0.202]$ (---) in 0.01 M NaCl/0.01 M sodium cacodylate (pH 7).

$40°$ to $50°$. A change in the tilt angle by $10°$ has a relatively strong effect only on the h_+/h_0 ratio as evidenced by the line-shape simulations. Since a reduction of the double bond converts the two carbons of the tether closest to the pyrimidine from trigonal planar to tetrahedral, it can be expected that this will affect the tilt angle. Most important in these simulations is the $\tau_\perp$ value, which reflects base motion arising from tilting and torsion of base pairs and base twisting. As is apparent from the simulation parameters, $\tau_\perp$ is of the order of 4 ns, a value previously reported for all base-paired spin-labeled thymidine analogues so far studied.

Note the existence of subtle differences in the ESR line-shapes of $(dA)_n(dT,DUPAT)_n$ and $(dA\text{-}dT,DUPAT)_n$, which were also observed between $(dA)_n(dT,DUAT)_n$ and $(dA\text{-}dT,DUAT)_n$ (for differences, see h_+/h_0 and h_-/h_0 ratios in Table I). The differences are, however, too small to be characterized with the motional model presently used. It is important to stress that both probes, DUAT and DUPAT, indicate the presence of small variations of thymidine dynamics between $(dA)_n(dT)_n$ and $(dA\text{-}dT)_n$ duplexes.

The effect of line-shape broadening at a high DUAVAP incorporation in double-stranded $(dA\text{-}dT,DUAVAP)_n$ is shown in Figure 8. It was noticed that the centerfield peak height of the ESR spectra of DUAVAP-containing polymers levels off at an incorporation level of 8–10%, whereas at a lower label incorporation the signal intensity directly reflects the amount of incorporated nitroxides. Since the nitroxide radicals are microscopically concentrated on the polymer lattice, it is not suprising that intensity loss and line-shape broadening are noticeable at a relatively low absolute nitroxide concentration. In the case of the single-stranded $(ls^4U,C)_n$, ESR line-shape broadening already became noticeable when the incorporation was greater than a few percent. It was shown that this broadening can be attributed to Heisenberg spin exchange (Kao & Bobst, 1986) by using the formalism applied by Meirovitch (1983) to evaluate the efect of nitroxide–nitroxide interactions on the ESR line shape.

DISCUSSION

Previously, a wide variety of position 4 and 5 nitroxide-labeled uridine and deoxyuridine derivatives have been incorporated into single-stranded nucleic acids with enzymes which were template independent. These spin-labeled single strands were annealed with complementary sequences to form double strands, and both systems were used for biochemical and biophysical studies. The versatility of these systems was limited to a certain degree because they could not address problems of sequence dependence. While all the spin-labeled substrates using either an oxygen or a sulfur linkage into position 5 of the pyrimidine ring for the nitroxide-containing tether were good substrates for template-independent enzymes such as polynucleotide phosphorylase (Bobst & Torrence, 1978) and

terminal deoxynucleotidyltransferase (Toppin et al., 1983), they served as poor substrates for template-dependent enzymes. We reported with pppDUGT, which contains an oxygen linkage, and the template-dependent enzyme AMV reverse transcriptase an incorporation of one spin-labeled base per 10^3 bases (Warwick et al., 1983). ppp-DUTT, which has a sulfur linkage, was found to bind more tightly to AMV reverse transcriptase than dTTP (Warwick et al., 1982).

All the analogues used for this study use a carbon linkage in position 5 of the pyrimidine ring for the attachment of the tethered nitroxide. Such a linkage helps to make the spin-labeled nucleotide a good substrate for pol I, although some specific properties of the tether must also be taken into account for designing the best possible analogue for pol I. Pol I has in the past been shown to have a limited ability to incorporate unnatural purine and pyrimidine base analogues (Kornberg, 1965) as well as nucleotides modified in their sugar moiety (Letsinger et al., 1976). More recently, this phenomenon has been extensively used to synthesize nucleic acid affinity probes (Langer et al., 1981), to make light-sensitive DNA (Evans & Haley, 1987), and to introduce unique damages into DNA (Ide et al., 1987). It can be seen from the kinetic data in Figure 3 that ppp-DUAVAP, ppp-DUAP, and ppp-DUAT are all good substrates for pol I. $(dA-dT)_n$-directed incorporation into $(dA-dL)_n$ proceeded at rates which were somewhat lower than with the natural substrate TTP. ppp-DUAVAP was found to be the best substrate with an initial rate similar to that observed with TTP. Incorporation of ppp-DUAVAP and ppp-DUAT by nick translation into λ phage DNA was also successful, but a less pronounced discrimination in the incorporation rates of these two spin-labeled substrates was noticed with the viral DNA as a template (data to be reported elsewhere).

It is interesting to note that a reduction of the double bond in the tether of ppp-DUAT, which yields the substrate ppp-DUPAT, changes the ability of the spin-labeled nucleotide to act as a substrate significantly. ppp-DUPAT is not a good substrate for pol I. This seems to imply that the double bond in ppp-DUAT is important possibly because it provides some rigidity to the tether which restricts conformational variabilities of the tether part closest to the pyrimidine base. A reduction of the double bond may allow the tether to assume conformations which are considerably less favorable for the DNA polymerase/substrate/template/primer complex. The detailed mechanism by which pol I copies DNA remains obscure. On the basis of a model proposed by Kornberg (1980), the orientation of the 5-substituent within the complex is determined. It is interesting to note that Otvos et al. (1987) have also noticed that the absence of a double bond in the C_1-C_2 of the 5-substituent can reduce the relative initial incorporation rate with pol I as for instance in the case of 5-pentyl or 5-hexyl versus 5-pentenyl or 5-hexenyl dUTP incorporation.

From Figure 4, it appears that the kinetics of polymerization in a mixed TTP/dLTP (1:1) system are a composite of the rates observed with the two individual components. This observation is in full agreement with the results reported by Sagi et al. (1977), who studied the incorporation of various 5-alkyl-dUTP compounds with a $(dA-dT)_n$ template using pol I. As suggested by these authors for their 5-alkyl-dUTP system, it is likely that in a mixed TTP/dLTP polymerization the resulting product is a randomly mixed copolymer.

A comparison of the spectra in Figures 2 and 6 and the line-shape parameters given in Table I both indicate that the analogues DUAT, DUAP, and DUPAT are very sensitive to the dimension of the major groove of the duplex. Bobst et al.

(1984b) have shown with the spin-labeled analogue DUMPT, which contains the same number of bonds in its tether as DUAT, DUAP, and DUPAT, that the nitroxide ring resides within the major groove of the duplex. On that occasion, it was also shown that an extension of the tether length by just one additional carbon, as in the case of the analogue DUMBT, moves the nitroxide ring outside of the sterically restricted environment of the major groove. Thus, such probes, if strategically placed in a DNA sequence, could experimentally verify the proposal by Drew and Travers (1985a) that sequence-dependent digestion of DNA by nucleases is caused by variations in the helix conformation. Namely, it is believed that the two grooves of a DNA double helix may vary in their size according to the helix conformation. If the minor groove is getting more narrow due to a particular A- and T-containing sequence, then the major groove on the other side of the helix will be wider, and vice versa (Drew & Travers, 1985b). Thus, a widening of the major groove in a sequence containing DUAT, DUAP, or DUPAT would result in some narrowing of the ESR line shape, since these labels are just barely within the sterically restricted domain of the major groove of a B-DNA duplex as has been experimentally shown with DUMPT and DUMBT (Bobst et al., 1984b).

Table I also shows that DUAVAP is not very sensitive in reflecting its incorporation into a nucleic acid lattice. This is due to its tether which is even longer than the one in DUMBT, and causes the nitroxide moiety to reside outside of the major groove as was observed with incorporated DUMBT. Although both DUMBT- and DUAVAP-containing polymers allow the monitoring of conformational transitions from single to double strands, the line-shape changes are far more subtle than in the case of spin-labeled nucleic acids with a shorter tether forcing the nitroxide to remain in the major groove. Since DUAP-, DUAT-, and DUAP-containing polymers are very sensitive in reflecting a single-strand to double-strand transition, they should be particularly suitable for application as hybridization probes. Such hybridization probes can be obtained by nick translation with pol I or end replacement with T4 polymerase (to be reported elsewhere). They offer the advantage of considerable shelf life, and hybridization detection by ESR may be readily automated.

For many years, spin-labeled nucleic acids have provided experimental evidence for base-dependent rapid motions in nucleic acids. More recently, a simple motional model was developed which was based on up-to-date ESR theory for the analysis of spin-labeled nucleic acid spectra (Kao et al., 1983; Kao & Bobst, 1985). Although the motional model described may appear simplified, since it involves only an effective tilt angle for the principle diffusion axis and two dynamic parameters ($\tau_\parallel$ and $\tau_\perp$), it essentially represents the limit to which a single ESR spectrum can be analyzed. It was shown by Campbell et al. (1979) how complex models of motional dynamics with many degrees of freedom will tend to reduce down to just a few measurable parameters. It is interesting to note that the motional values for base motions in nucleic acid duplexes obtained by ESR with spin-labeled nucleic acids are in excellent agreement with more recent NMR studies (Assat-Munt et al., 1984; Behling & Kearns, 1986).

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