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Enzymatic sequence-specific spin labeling of a DNA fragment containing the recognition sequence of *Eco*RI endonuclease

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Deoxyuridine analogs spin labeled in position 5 have been enzymatically incorporated sequence specifically into an oligodeoxyribonucleotide to form a spin-labeled 26-mer. The 26-mer contains the *Eco*RI-binding site and two labels which are located symmetrically close to the binding site. The labels are separated from one another far beyond the Heisenberg spin-exchange distance. The local base motion as determined by ESR spectroscopy is of the order of 4 ns in the oligonucleotide duplex. This is the same value as reported earlier for local T motions in polynucleotide duplexes, thereby providing direct experimental evidence that the ESR line shape of spin levels covalently attached to nucleic acids depends primarily on the local dynamics of the nucleic acid building blocks.

Spin-labeled oligonucleotide; Base dynamics; DNA polymerase; Klenow fragment; Melting profile; ESR

1. INTRODUCTION

Spin-labeled nucleic acids have been used successfully for many years to study some of the properties of nucleic acids by ESR [1-4] spectroscopy. The incorporation of spin labels into nucleic acids can be achieved by chemical or enzymatic means. This laboratory has developed a variety of position 4 and 5 nitroxide-labeled uridine and deoxyuridine derivatives to serve as substrates for nucleic acid-synthesizing enzymes. Either template-independent enzymes such as polynucleotide phosphorylase [5-7] and terminal deoxynucleotidyltransferase [8], or template-dependent enzymes such as reverse transcriptase from avian myeloblastosis virus [9] and DNA polymerase I from *E. coli* [10] were used for this purpose. While it was also possible to incorporate nitroxide radicals into nucleic acids with defined sequences by nick-translation to obtain hybridization probes (to be reported elsewhere), the enzymatic approaches reported so far only allowed us to replace randomly thymidine residues with spin-labeled analogs.

For studies which involve nucleic acid sequence-specific binding proteins, it was necessary to find means of incorporating spin labels into a nucleic acid site specifically with respect to its sequence. In principle, this can be achieved by the technique of oligonucleotide synthesis, but the standard chemistry and purification procedures required by this methodology will irreversibly oxidize many of the nitroxide radicals, and we find it difficult to isolate pure spin-labeled oligonucleotides by this route (to be published elsewhere). Enzymatic incorporation, on the other hand, does not interfere with the chemical reactivity of the nitroxide, and we report the enzymatic synthesis of a sequence specifically spin-labeled oligonucleotide containing the *Eco*RI endonuclease recognition sequence.

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2. MATERIALS AND METHODS

The 17-mer 5'-CCCCACCCGCGAATTCG was synthesized on an Applied Biosystems 380 A DNA synthesizer using phosphoamidate chemistry and the 17-mer was characterized by gel electrophoresis. The non-radioactive triphosphates were obtain-

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ed from Pharmacia, [8-^{3}H]deoxyguanosine 5'-triphosphate from ICN and Bio-Gel P30 gel from Bio-Rad. The large DNA polymerase (Klenow) fragment was purchased from Promega. The spin-labeled thymidine triphosphate analog (dLTP) ppp-DUAT (**I**) (for spin-labeled structures see fig.1) was synthesized according to a procedure of this laboratory [11]. The synthesis of the other two dLTPs used here, ppp-DUAP (**II**) and ppp-DUAVAP (**III**), will be published elsewhere.

2.1. *Preparation of radiolabeled 26-mer (V)*

The end filling of the partially complementary 17-mer (**IV**) was carried out in propylene tubes with 0.2 nmol **IV**, 4 nmol dTTP or 4 nmol ppp-DUAP (**II**) or 4 nmol ppp-DUAVAP (**III**), 30 nmol dGTP (spec. act. 17 cpm/pmol) and 6.6 U Klenow fragment in a total volume of 102 μl containing 0.14 M Tris-HCl, 0.05 M NaCl, pH 7.8, 0.014 M $MgCl_2$, 1.4 mM dl-dithiothreitol and 28 μg/ml bovine serum albumin. The mixture was incubated at room temperature, and 5-μl aliquots were withdrawn at appropriate time intervals and spotted onto Whatman DE 81 filters. The filters were washed 6 times with 0.5 M Na_2HPO_4, 3 times with water, and twice with 95% ethanol, dried, and then counted in a toluene-based scintillation fluid (PPO/POPOP).

2.2. *Preparation of spin-labeled 26-mer (V)*

The same end filling protocol used for the radiolabeled 26-mer was applied to synthesize spin-labeled 26-mers by fully substituting TTP with either ppp-DUAT (**I**) or ppp-DUAP (**II**). The amount of the individual components was increased by a factor of 4 to isolate spectroscopic quantities of the spin-labeled 26-mer (**V**). The enzymatic reaction was stopped after 90 min with 50 μl of 0.3 M EDTA and the 26-mer was separated from unincorporated nucleotides with Bio-Gel P-30 gel filtration using as elution buffer 0.04 M NH_4HCO_3, pH 7.5, 10% ethanol. About 0.4 nmol 26-mer (**V**) containing for L either T, DUAT (**I**) or DUAP (**II**) was isolated by this procedure.

The ESR spectra were recorded with a Varian E-104 Century Series spectrometer interfaced with an Apple II plus microcomputer [12] using the parameters in [7]. Simulation of the ESR spectrum of the DUAT-containing 26-mer was performed with the motional model discussed in [8,10]. The thermal denaturation studies followed by UV absorbance (A) at 260 nm were performed in a Gilford 250 spectrophotometer with a Gilford 2527 thermoprogrammer [13].

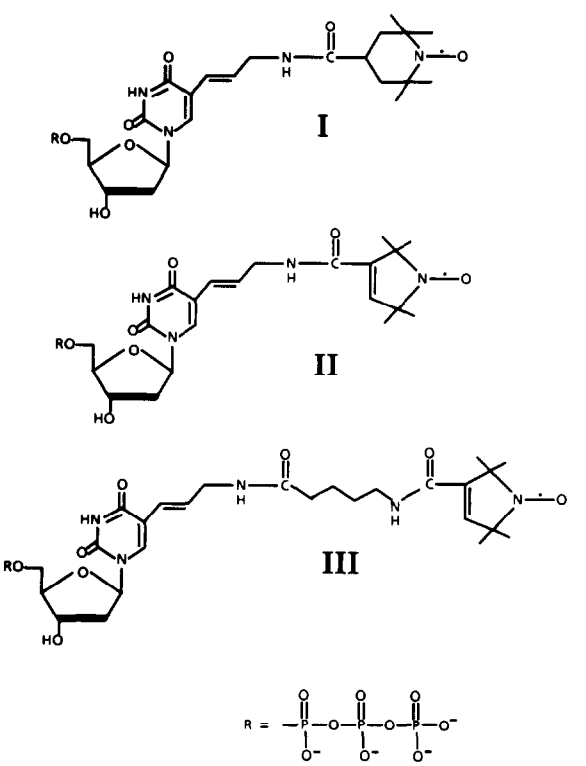

Fig. 1. Chemical structures of the nitroxide-labeled thymidine analogs ppp-DUAT(**I**), ppp-DUAP(**II**) and ppp-DUAVAP (**III**).

3. RESULTS AND DISCUSSION

Fig.2 shows the scheme of the enzymatic reaction used for the end filling of the partially complementary 17-mer (**IV**) with the large DNA polymerase (Klenow) fragment. The reaction leads to the formation of the 26-mer (**V**) where the sequence-specifically incorporated L represents either T, or one of the spin-labeled analogs **I–III**.

The kinetics of dGMP incorporation shown in fig.3 was determined by monitoring the [8-^{3}H]-dGMP uptake with TTP, ppp-DUAP (**II**) or ppp-DUAVAP (**III**) as substrate for the complementary base A. As a control, the reaction was followed in the absence of any thymidine or thymidine analog. The data show that both spin-labeled analogs act as an excellent substrate. Essentially the same incorporation kinetics is observed with **II** and **III** as with T. Analog **I** was not tested but based on its incorporation determined with DNA polymerase I on alternating copolymers [10], it should show similar incorporation behavior to **II**. In the absence of T only about half of the total amount of dGMP is incorporated as can be expected from fig.2.

The potential perturbation effect of L on the thermodynamic stability of the 26-mer structure was evaluated by temperature-dependent UV measurements at 260 nm, a wavelength which allows monitoring of both d(AT) and d(GC) base pairs [14]. The melting profile of the unlabeled 26-mer (**V**) (L = T) is compared with the profiles obtained with 26-mer (**V**) (L = DUAP) and 26-mer (**V**) (L = DUAT). As can be seen from fig.4, the 3

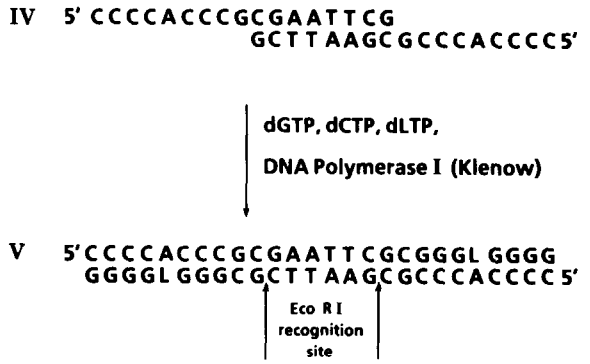

Fig. 2. General scheme for the sequence-specific introduction of the spin-labeled residues L into the partially complementary 17-mer (**IV**) to synthesize the 26-mer (**V**) with Klenow fragment.

profiles are the same. None of the 3 distinct transitions are affected by the presence of the spin label. The transition with a T_m value of 50°C reflects the melting process of the d(AT) region, while the high-temperature transitions with T_m values of 65 and 80°C correspond to the melting of d(GC) base pairs. It is likely that the melting of the d(GC) base pairs occurs in two steps, since one can distinguish between alternating G-C stretches and homopolymer G and C regions in the 26-mer (**V**). The similarity in melting profiles of unlabeled and labeled 26-mer provides good evidence that a tethered nitroxide radical substituted in position 5

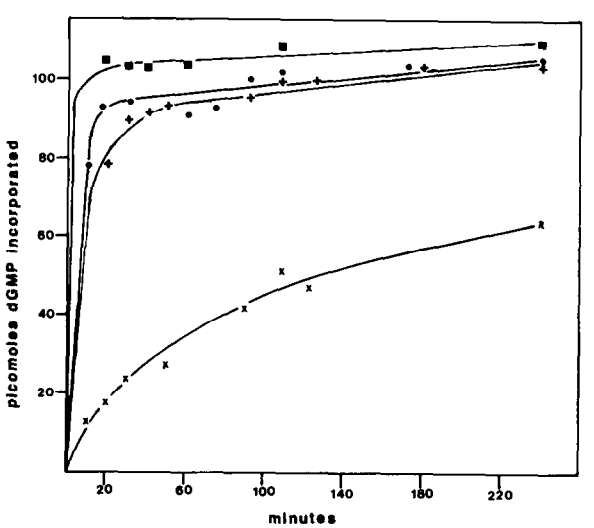

Fig. 3. Incorporation of dGMP into 17-mer (**IV**) as a function of time. Reactions contain (+) TTP, (○) ppp-DUAVAP, (■) ppp-DUAP, and (×) no thymidine or thymidine analog.

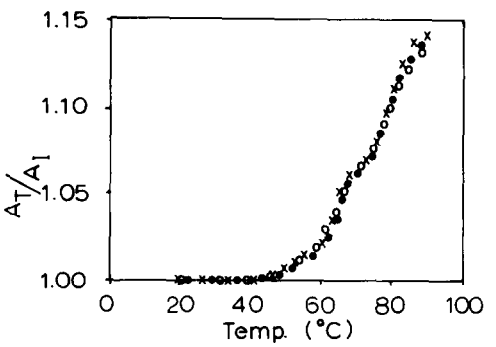

Fig. 4. Normalized UV absorbance-temperature profiles at 260 nm of 26-mer (**V**) (L = T) (×), 26-mer (**V**) (L = DUAT) (○), and 26-MER (**V**) (L = DUAP) (●) in 0.01 M sodium cacodylate, pH 7, 0.01 M NaCl, 0.1 mM EDTA at a nucleotide residue concentration of (3.5 - 5 × 10^{-5} M.

of deoxyuridine causes no structural mismatches. It had been shown earlier that mismatched bases will strongly affect the thermodynamic stability of double helices [15].

Fig.5A shows the experimental and computer-simulated ESR spectrum of the 26-mer (**V**) with two DUAT labels separated from one another far beyond the Heisenberg spin-exchange distance [16]. A related ESR spectrum is also observed with a DUAP-containing 26-mer (fig.5B). The spectrum of fig.5A is similar to that reported earlier for the

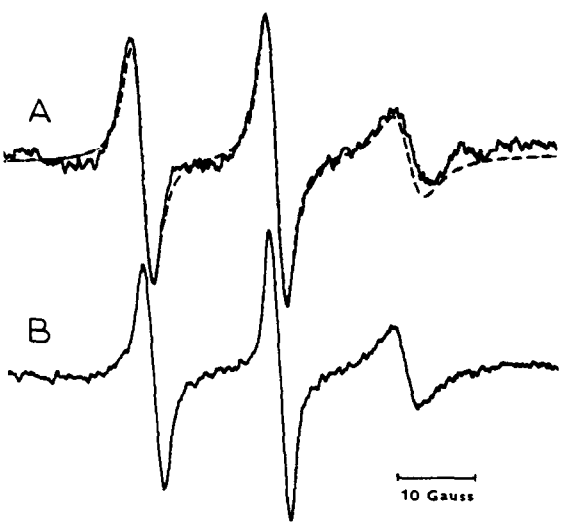

Fig. 5. (A) Experimental (——) and computer-simulated (- - -) ESR spectra of 0.3 nmol 26-mer (**V**) (L = DUAT); (B) experimental ESR spectrum of 0.3 nmol 26-mer (**V**) (L = DUAP). ESR spectra measured in 0.01 M sodium cacodylate, pH 7, 0.01 M NaCl, 0.1 mM EDTA.

polymer duplex (DUAT,dT)$_n$(dA)$_n$ [17]. The computer simulation gives a good fit with the same motional model and the same parameters reported earlier for the polymer system [7]. The only difference in simulation parameters between the polymer and oligomer system is the value of the tilt angle, which was 40° for the polymer, while for the 26-mer a better fit was obtained with a slightly higher value of 45°. The tilt angle is used here as defined in [7,18] and is believed to reflect some of the sterically restricted environment of the major groove containing the tethered nitroxide ring. There is some evidence from sequence-dependent digestion of DNA by nucleases that the dimensions of the major and minor grooves vary in size according to the nucleic acid sequence [19]. It is to be expected that the steric environment of the major groove displays subtle geometric differences between the polymer and 26-mer duplex, since L has T residues as nearest neighbors in (DUAT, dT)$_n$(dA)$_n$ and G residues in the 26-mer.

The local base motion of L is of the order of 4 ns in the 26-mer, a value reported previously for local T motions in polynucleotide duplexes [10,17]. This is the first direct experimental evidence for the hypothesis that the nucleic acid spin-labeling approach described here and in previous publications from our laboratory reports local base mobility, and consequently does not primarily depend on the size of the nucleic acid duplex. The base motion values determined by NMR were obtained on oligonucleotide duplexes [20,21] and are in complete agreement with the ESR data obtained on oligo- and polymer systems.

From the scheme shown in fig.2 it is obvious that the location of the probe L in the 26-mer can be readily changed by selecting an appropriate 17-mer. Thus, a whole series of sequence specifically labeled 26-mers containing the *Eco*RI endonuclease recognition site with strategically placed reporter groups near the binding site can be constructed with the described approach. The synthesis of such sequence-specifically spin-labeled oligonucleotides is presently in progress in view of X-ray data showing that the *Eco*RI endonuclease-DNA recognition complex contains localized disruptions of the double-helical symmetry [22]. It has been proposed that these disruptions may have structural consequences which could propagate over long distances. Strategically placed L probes will be ideally suited to evaluate the propagation effect caused by the protein-nucleic acid binding process.

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