13. Template-Dependent Incorporation of Spin-Labeled Thymidine Analogs into Viral DNA

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(16.XI.88)

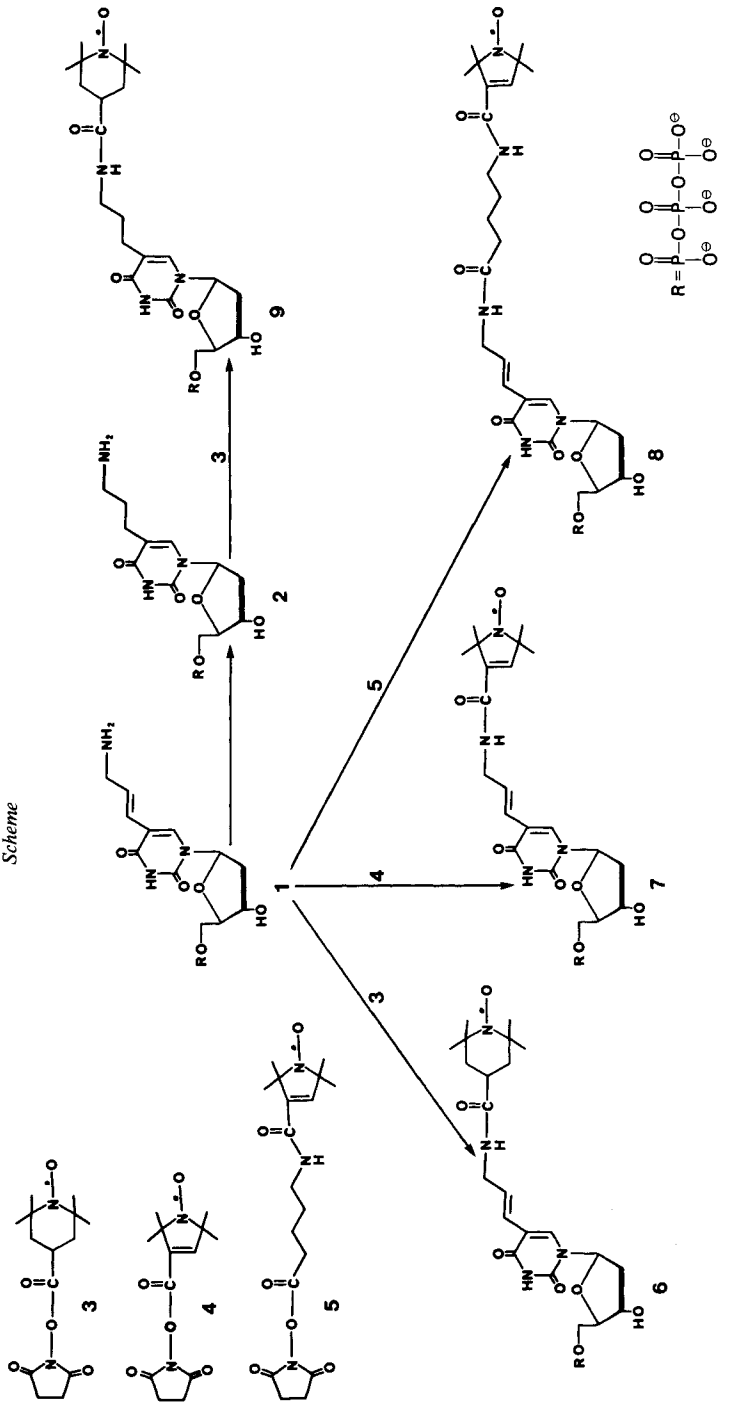
The synthesis of novel $pppU_d$ analogs substituted at position C(5) with tethered nitroxide radicals is reported. It is shown that these analogs can serve as good substrates for *E. coli* DNA polymerase (Pol I) and T-4 DNA polymerase using lambda-phage DNA as template. The template-dependent incorporation of the substrates was established by radio-labeling and ESR experiments.

Introduction. – DNA homopolymers containing randomly incorporated spin-labeled deoxyuridine analogs were previously obtained by copolymerizing spin-labeled deoxyuridine triphosphates and $pppT_d$[1]) with a template-independent nucleic-acid polymerase such as TDT [1] [2]. More recently, we showed that some spin-labeled substrates $pppL_d$ such as ppp-opip^5U_d (**6**, R = ppp), ppp-opipr^5U_d (**9**, R = ppp), ppp-opyp^5U_d (**7**, R = ppp), and ppp-opype^5U_d (**8**, R = ppp) can also be used by Pol I to form the spin-labeled alternating copolymers $(A_d\text{-}T_d, L_d)_n$ [3] [4]. Here, we report the chemical synthesis of **7**–**9** (the synthesis of **6** was published in [3]) and show that these analogs can be incorporated into lambda-phage DNA. Compound **8** was incorporated into Hind-III-restriction fragments of lambda-phage DNA by end replacement with T-4 DNA polymerase and compounds **6**, **8**, and **9** into lambda-phage DNA by nick-translation. The ESR signals of the spin-labeled viral DNA's can be used to determine the hybridization state of the viral DNA. Therefore, these spin-labeled compounds are particularly suitable for safe hybridization assays with ESR as detection device.

Usually, nucleic acids are labeled with radioactive ^{32}P to give sensitive hybridization probes. Disadvantages of radio-labeling typically entail the intricate nature of autoradiographic detection methods, the instability of isotopes such as ^{32}P, and problems with radioactive contamination. More recently, non-isotopic probing techniques have been developed. Some rely on the enzymatic incorporation of biotin-labeled nucleoside triphosphates and the subsequent affinity interaction by conjugates of avidin or streptavidin with enzyme molecules [5] [6]. While the sensitivity level of ESR hybridization assays

[1]) *Abbreviations:* $pppT_d$, thymidine triphosphate; TDT, terminal deoxynucleotidyl transferase; Pol I, *E. coli* DNA polymerase I; DNAse I, deoxyribonuclease I; L_d, spin-labeled deoxyuridine analog; pL_d and $pppL_d$, mono- and triphosphate of L_d, respectively; opip^5U_d (formerly DUAT), 5-[3-(2,2,6,6-tetramethyl-1-oxylpiperidine-4-carboxamido)prop-1-enyl]-2′-deoxyuridine (**6**, R = H); opipr^5U_d, 5-[3-(2,2,6,6-tetramethyl-1-oxylpiperidine-4-carboxamido)propyl]-2′-deoxyuridine (**9**, R = H); opyp^5U_d, 5-[3-(2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxamido)prop-1-enyl]-2′-deoxyuridine (**7**, R = H); opype^5U_d, 5-{3-[5-(2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrol-3-carboxamido)pentanamido]prop-1-enyl}-2′-deoxyuridine (**8**, R = H); PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin, SDS, sodium dodecyl sulfate.

Scheme



at present is still lower than that of the methods currently in use, it offers the advantage of being readily automated.

Results and Discussion. – The *Scheme* shows the strategy employed for the synthesis of the spin-labeled nucleoside triphosphates. The modified nucleotide **1** was obtained by minor modifications of a published procedure [7] which included an HPLC purification step and no hydrogenation step to give **2**. Although **1** did contain some contaminants before the condensation reactions with either **3**, **4**, or **5**, it was found to be more economical to purify the end products by HPLC. The nucleotides **6**–**9** thus obtained were characterized by ^{1}H-NMR spectroscopy (see *Table*).

As was the case in the polymerization system using a $(A_d\text{-}T_d)_n$ template [4], the enzymatic incorporation reactions can be divided into two groups. In the first group, the spin-labeled thymidine analog is the only source of a thymidine-like triphosphate and allows us to assess the inherent ability of the probe to be incorporated into the lattice with the polymerase by monitoring the kinetics of p[^{3}H]A_d incorporation. In the second group, the nick-translation reaction is carried out on a larger scale either with modified triphosphate analogs as the only source of a thymidine-like compound or with mixtures of $pppL_d$ and $pppT_d$. The larger-scale synthesis makes the isolation of nick-translated nucleic acids possible for spectroscopic studies by ESR.

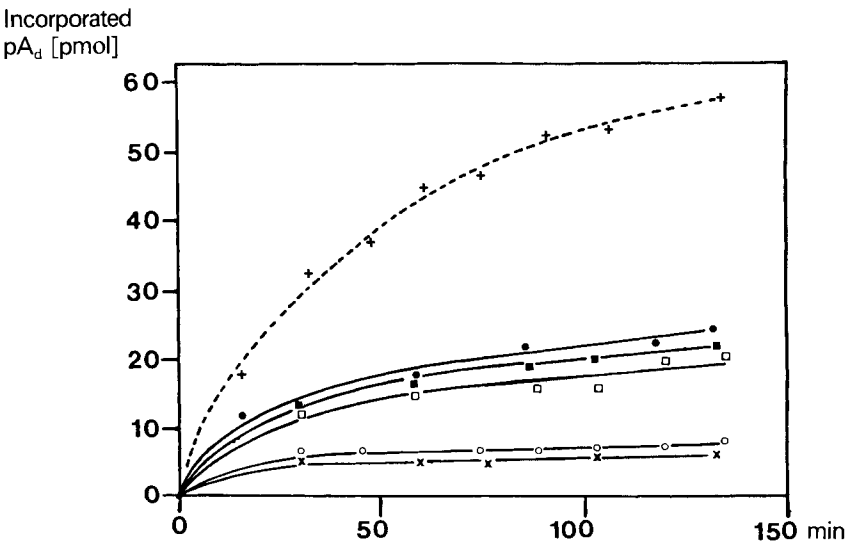


Fig. 1. *Incorporation of p[^{3}H]A_d into lambda-phage DNA by nick-translation as a function of time.* Reaction mixture contains either $pppT_d$ (+), ppp-opype^5U_d (**8**; ●), ppp-opyp^5U_d (**7**; ■), ppp-opip^5U_d (**6**; □), or ppp-opipr^5U_d (**9**; ○). (×) is a control without thymidine or thymidine analog.

Fig. 1 shows the kinetics of nick-translation reactions with the thymidine analogs **6**–**9** without any $pppT_d$, as well as some control kinetics with only $pppT_d$ or without any $pppT_d$ and thymidine analogs. The incorporation of p[^{3}H]A_d, which can be considered to reflect the incorporation rate of $pppL_d$, clearly shows that the probes **6**–**8** are all well incorporated into lambda-phage DNA. Triphosphate **9** does also support some detect-

able polymerization over a no-thymidine control, though the incorporation of **9** occurs at levels substantially below those of the other related compounds. It is interesting to note that the relative abilities of the various probes to be incorporated into lambda-phage DNA appear to be consistent with the observations made in the $(A_d\text{-}L_d)_n$ incorporation system [4]. However, the differences in the incorporation efficiencies of **6–8** are by far less pronounced with lambda-phage DNA than with $(A_d\text{-}L_d)_n$. *Letsinger et al.* [8] made a similar observation when they reported that ΦX-174 DNA served as a much better template for the misincorporation of nucleotide analogs than $(A_d\text{-}T_d)_n$.

For the ESR measurements showns in *Fig. 2b*, lambda-phage DNA has been nick-translated on a larger scale with a mixture of 78% of $pppL_d$ and 22% of $pppT_d$. Under such conditions, one determines an ESR lineshape for the spin-labeled viral DNA which is characteristic for a double-stranded B-DNA system. The ESR spectrum is similar to that determined for (A_d-T_d, opip^5U_d)$_n$ [4] or (opip^5U_d, T_d)$_n$·(A_d)$_n$ [9]. For comparative purposes, the experimental and computer-simulated ESR spectra of (opip^5U_d, T_d)$_n$·(A_d)$_n$

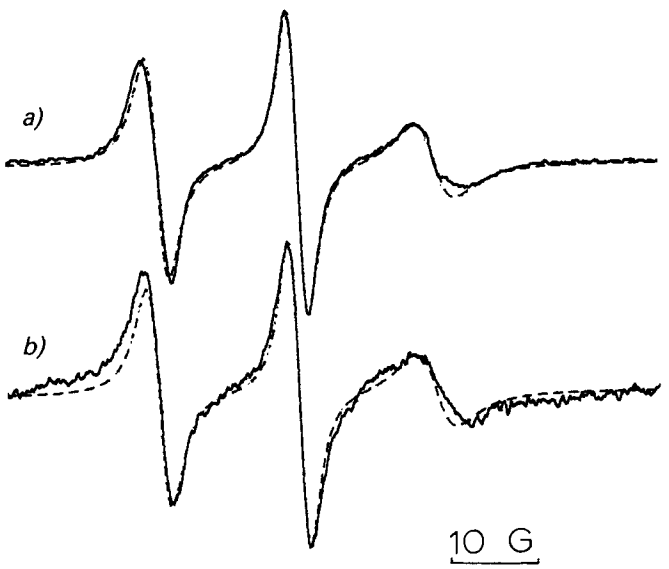


Fig. 2. *Experimental* (——) *and computer-simulated* (– – –) *ESR spectra of* a) $1.9 \cdot 10^{-4}$ M *(opip^5U_d,T_d)$_n$·(A_d)$_n$* (opip^5U_d/T_d = 0.02) *in 10 mM NaCl/10 mM sodium cacodylate* (pH 7.0), *and* b) *4 µg of lambda-phage DNA nick-translated with 72% of ppp-opip^5U_d* (**6**) *and 28% of pppT_d in 10 mM NaCl/10 mM* Tris-*HCl* (pH 7.9)

are shown in *Fig. 2a*. The same parameters used for the simulation of the ESR spectrum of (opip^5U_d, T_d)$_n$·(A_d)$_n$ are used to simulate the spectrum shown in *Fig. 2b* of the spin-labeled viral DNA. A good fit is also observed with the viral DNA suggesting that the probe reflects the local base dynamics of a B-DNA conformation present under these conditions in lambda-phage DNA. It should be noted that in larger-scale reactions which use only $pppL_d$ in place of $pppT_d$, the resulting lambda-phage-DNA ESR spectrum (spectrum not shown) exhibits significant broadening due to *Heisenberg* spin exchange [10] [11]. This is not surprising, because lambda-phage DNA contains many stretches in its sequence where two or more thymidine residues appear in a row.

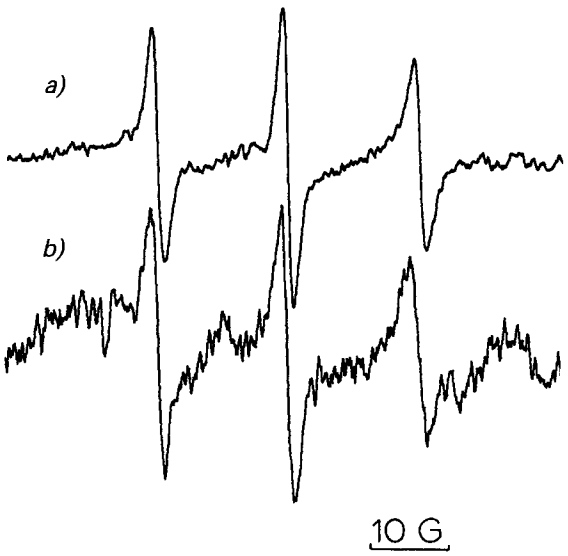


Fig. 3. *ESR spectra of* a) *2 μg of lambda-phage DNA nick-translated with ppp-opype^5U_d and Pol I, and* b) *of 5 μg of Hind-III-digested lambda-phase DNA end-labeled with ppp-opype^5U_d and T-4 DNA polymerase.* Spectra are recorded in 1M NaCl/0.1M sodium citrate (pH 7.0) with 0.5% of SDS.

Fig. 3 shows ESR spectra of **8** incorporated either by nick-translation into lambda-phage DNA or introduced into Hind-III-restriction fragments by T-4 DNA polymerase. Both systems give similar ESR spectra which closely resemble the ESR spectrum reported earlier for the spin-labeled alternating copolymer $(A_d\text{-}T_d, \text{opype}^5U_d)_n$ [4].

In conclusion, spin-labeled thymidine analogs have been enzymatically incorporated into lambda-phage DNA or into its Hind-III-restriction fragments. The ESR spectra of the spin-labeled viral DNA's are in agreement with ESR data obtained earlier on spin-labeled nucleic acids consisting of homopolymers or alternating copolymers.

This investigation was supported in part by a grant from the U.S. Public Health Service (GM 27002).

Experimental Part

General. All chemicals not specified were of reagent grade or better and were purchased from *Sigma Chemical Co., Aldrich Chemical Co., Fischer Scientific Co., J. T. Baker Chemical Co., MCB Manufacturing Chemists Inc.*, or *Kodak Laboratory Chemicals.* Pol I/DNAse I consisting of Pol I (0.4 U/μl) and DNAse I (40 pg/μl), T-4 DNA polymerase, lambda-phage DNA, and Hind-III-digested lambda-phage DNA were all bought from *Bethesda Research Laboratories, Inc.* The synthesis of 5-(3-aminoprop-1-enyl)-2′-deoxyuridine 5′-triphosphate (**1**, R = ppp) and 2,5-dioxopyrrolidin-1-yl 2,2,6,6-tetramethyl-1-oxylpiperidine-4-carboxylate (**3**) and their condensation to 5-[3-(2,2,6,6-tetramethyl-1-oxylpiperidine-4-carboxamido)prop-1-enyl]-2′-deoxyuridine 5′-triphosphate (**6**, R = ppp) has been described [3]. The 2,5-dioxypyrrolidin-1-yl 2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxylate (**4**) is commercially available from *Kodak Laboratory Chemicals.* Anion-exchange chromatography: at r.t. using *DEAE-Sephadex A-25 (Sigma)* packed in a 15 cm × 16 mm column. Prep. and anal. paper chromatography: *Whatman 3MM* and *Whatman No. 1*, resp.; elution with abs. EtOH/1M $AcONH_4$ 7:3 (*v*/*v*). Prep. and anal. TLC: 20 × 20-cm *Analtech Uniplate 2000 micron* silica gel *GF* and 10 × 2.5 cm *Analtech Uniplate 250 micron* silica gel *GF* plates resp. HPLC purification: *Bioanalytical System Instrument* with a *Waters μ-Bondapak C_{18}* column using a step gradient of 50 mM $(NH_4)_3PO_4$ (solvent system *A*) and MeOH/H_2O 1:1 (*v*/*v*) (solvent system *B*); after

sample injection, *A* was applied for 100 s, followed by elution with 65% *A* and 35% *B*. The extinction coefficients of the nucleoside triphosphates were determined by phosphate analysis [12]. Purification of spin-labeled nucleic acids for ESR analysis was accomplished by exclusion chromatography over a *Sephadex-G-50* column with 150 mM NaCl/15 mM sodium citrate (pH 7) with 0.1% (*w/v*) of SDS. The polymer-containing fractions were concentrated by centrifugation with an *Amicon K-30 Centricon* filtration device, and the buffer was exchanged by washing through the *Centricon* filter 3 times with 1.5 ml of a 10 mM *Tris*-HCl/10 mM NaCl buffer (pH 7.9). ESR spectra: *Varian E-104* spectrometer interfaced to an *Apple II* computer. NMR spectra: *ca.* 0.15 mg of nucleoside triphosphate dissolved in 400 µl of D_2O; *Nicolet NTC 300 FT* instrument. MS: direct inlet; *Hewlett-Packard 5995* GC/MS.

5-(3-Aminopropyl)-2′-deoxyuridine 5′-Triphosphate (**2**, R = ppp). To **1** (R = ppp; 0.042 mmol) in H_2O (10 ml), 10% Pd/C (10 mg) was added. The mixture was saturated with H_2, stirred vigorously under a H_2-filled balloon for 4 h at r.t., and then filtered through *Celite*. The material (*ca.* 100% conversion) was further purified by *DEAE-Sephadex* chromatography with a linear gradient of 0.1M to 0.5M triethylammonium hydrogen carbonate (pH 7.5).

2,5-Dioxopyrrolidin-1-yl 5-(2,5-Dihydro-2,2,5,5-tetramethyl-1-oxyl-1H-pyrrole-3-carboxamido)pentanoate (**5**). To 117 mg (1.0 mmol) of 5-aminopentanoic acid in 5 ml of H_2O saturated with Et_3N and adjusted with CO_2 to pH 10, 388 mg (1.0 mmol) of **4** in 4 ml of *N,N′*-dimethylformamide (DMF) were added. The mixture was stirred for 18 h at 50°, the solvent evaporated, and the residue purified by prep. TLC with MeOH/$CHCl_3$/conc. HCl 1:10:0.05 to give 300 mg of 5-(2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxamido)pentanoic acid as a yellow oil. This oil in 3 ml of DMF was added to 117 mg (1.0 mmol) of *N*-hydroxysuccinamide and 206 mg (1.0 mmol) of *N,N′*-dicyclohexylcarbodiimide in 3 ml of *N,N′*-dimethylcarbodiimide and stirred for 18 h. The white precipitate was centrifuged off and the solvent evaporated. Prep. TLC (MeOH/$CHCl_3$ 1:19) gave 103 mg (0.34 mmol) of **5** (R_f 0.48) as an amber oil. MS: 380 (M^+).

5-[3-(2,5-Dihydro-2,2,5,5-tetramethyl-1-oxyl-1H-pyrrole-3-carboxamido)prop-1-enyl]-2′-deoxyuridine Triphosphate (**7**, R = ppp). Condensation of **1** with **4** as outlined below gave **7** (R = ppp). R_f 0.48 (*Whatman No. 1*). HPLC: t_R 11.5 min. UV (pH 7.0): 241 (14100), 290 (8300). ^{1}H-NMR: *Table*.

Table. *^{1}H-NMR Chemical Shifts* (in ppm) *for H-Atoms of Unreduced Spin-Labeled Nucleotide Analogs Downfield from TMS*

Compound abbreviation	Pyrimidine H–C(6)	Sugar H–C(1′)	Tether olef. H	Nitroxide ring CH_3
ppp-opip^5U_d[a]) (**6**, R = ppp)	7.9 (br.)	6.3 (br.)	6.4 (br.)	1.25 (br.)
ppp-opipr^5U_d (**9**, R = ppp)	7.65 (br.)	6.3 (br.)	–	1.25 (br.)
ppp-opyp^5U_d (**7**, R = ppp)	7.9 (br.)	6.3 (br.)	6.4 (br.)	1.2 (br.)
ppp-opype^5U_d (**8**, R = ppp)	7.9 (br.)	6.3 (br.)	6.35 (br.)	1.2 (br.)

[a]) Note that ppm values reported for ppp-opip^5U_d in [3] are too small by 0.6 ppm due to erroneous calibration.

5-[3-(2,2,6,6-Tetramethyl-1-oxylpiperidine-4-carboxamido)propyl]-2′-deoxyuridine 5′-Triphosphate (**9**, R = ppp). Condensation of **2** with **3** as outlined below gave **9** (R = ppp). R_f 0.51 (*Whatman No. 1*). HPLC: t_R 10.4 min. UV (pH 7.0): 267 (8500). ^{1}H-NMR: *Table*.

5-{3-[5-(2,5-Dihydro-2,2,5,5-tetramethyl-1-oxyl-1H-pyrrole-3-carboxamido)pentanamido]prop-1-enyl}-2′-deoxyuridine 5′-Triphosphate (**8**, R = ppp). Condensation of **1** with **5** as outlined below gave **8** (R = ppp). R_f 0.6 (*Whatman No. 1*). HPLC: t_R 17.5 min. UV: 241 (16350), 290 (8300). ^{1}H-NMR: *Table*.

General Procedure for the Synthesis of **7**–**9**. To a soln. of 0.03 mmol of nucleotide **1** or **2** (R = ppp) in 2.5 ml of 0.1M sodium borate (pH 8.7), 0.06 mmol of the label ester **3**, **4**, or **5** in 0.3 ml of DMF were added. After 4 h of stirring, the mixture was diluted with H_2O and then loaded on a *DEAE-Sephadex* column. Compounds **7**–**9** were eluted with a gradient of 0.1M to 0.4M NH_4HCO_3 (pH 7.5). Coupling yield: *ca.* 80% for **7**–**9**, based on nucleotide.

Incorporation of p[^{3}H]A_d into Lambda-Phage DNA in the Presence of pppL$_d$. Reactions were carried out in polypropylene tubes, using 30 µl of a soln. of 0.1M *Tris*-HCl (pH 7.5), 0.01M $MgCl_2$, 0.001M DL-dithiothreitol, 22 µg/ml of nuclease-free BSA, 1.5 µg of lambda-phage DNA, 2.4 mmol each of $pppG_d$, $pppC_d$, p[^{3}H]A_d (spec. act. 650 Ci/mol) and either $pppT_d$ or $pppL_d$. Then, 10 µl of Pol I/DNAse 1 in 50 mM *Tris*-HCl (pH 7.5), 5 mM Mg$(OAc)_2$, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 50% (*v/v*) glycerol, and 100 µg/ml of nuclease-free BSA were added, and the mixture was incubated at 16°. At appropriate time intervals, 3-µl samples were spotted on *Whatman DE81* filters. The filters were counted in a toluene-based scintillation fluid (PPO/POPOP).

Incorporation of pL_d into Lambda-Phage DNA for ESR Measurements by Nick-Translation. The same protocol as for the preparation of tritiated product was used with the exception that the scale of the reaction was increased 3-fold. The reactions were stopped after 90 min by addition of 10 µl of 0.1M Na_2EDTA (pH 8.0) and 2.5 µl of 5% (*w/v*) SDS.

Incorporation of **8** (R = ppp) *into Hind-III-Restriction Fragments of Lambda-Phage DNA by End Replacement with T-4 DNA Polymerase for ESR Analysis.* A soln. (10 µl) of 50 mM *Tris*-AcOH (pH 7.9), 50 mM NaOAc, 10 mM $Mg(OAc)_2$, 0.5 mM DL-dithiothreitol, and 100 µg/ml of nuclease-free BSA was added to 10 µl of 0.3M NaOAc containing 5.5 µg of chloride-free Hind-III-digested lambda-phage DNA. This was incubated together with 5 units of T-4 DNA polymerase for 15 min at 37°, and then 7 µl of a soln. containing 3 nmol each of $pppA_d$, $pppG_d$, and $pppC_d$ were added together with 2 µl of an aq. soln. containing 3.8 nmol of **8** (R = ppp). After incubation at 37° for 35 min, the reaction was stopped by adding 10 µl of 0.3M Na_2EDTA (pH 8.0) and 2.5 µl of 5% (*w/v*) of SDS.

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