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EPR STUDY ON THE INTERACTION OF SPIN-LABELLED HYDRAZINE MUSTARD DERIVATIVES WITH DNA

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Abstract—The interaction of three spin-labelled compounds, derivatives of bis-(2-chloroethyl)-hydrazine (HMSL), *N*-methyl,*N*-chloroethyl-hydrazine (MCEHSL) and bis-(2-bromoethyl)-hydrazine (BEHSL) with DNA was studied by the method of electron paramagnetic resonance (EPR). It was found that HMSL (containing two chloroethyl groups) and MCEHSL (containing one chloroethyl group) gave spin-labelled dsDNA with identical strongly immobilized EPR spectra. The conclusion was drawn that only one of the alkylating groups of HMSL reacted with DNA. In contrast, the EPR spectrum of DNA spin-labelled with BEHSL was non-immobilized due to the strong destabilizing effect of this compound on the double helix. The extent of alkylation of DNA with the three hydrazine mustard derivatives was one and the same. It was found, however, that chloroethyl-containing derivatives (HMSL and MCEHSL) had an expressed base specificity and alkylated preferably the guanilic residues, and their bromo-analogue (BEHSL) did not show any base specificity and alkylated the bases of DNA at random.

Most of the hydrazine mustard derivatives are characterized by a strong cytostatic effect and they are widely applied in the chemotherapy of cancer. Although it is known that these substances suppress the replication of DNA, the right mechanism of their action remains unclear. A new attractive approach for studying the interaction of low mol. wt substances, including carcinogens and cytostatics [1, 2], with biopolymers is the method of electron paramagnetic resonance (EPR). This method was extensively exploited in studying the structure and conformation of RNA and proteins as well as the molecular organization of subcellular structures such as cell membranes, ribosomes etc. [3], but its application in the investigation of DNA has only just begun [4, 5].

The present paper aims to reveal the molecular mechanism of interaction of spin-labelled derivatives of bis-(2-chloroethyl)-hydrazine, *N*-methyl,*N*-chloroethyl-hydrazine and bis-(2-bromoethyl)-hydrazine with DNA by the method of EPR.

MATERIALS AND METHODS

Alkylating spin-label synthesis. (1) 3-[*N*,*N*-Bis-(2-chloroethyl) - carbohydrazide] - 2,2,5,5 - tetramethylpyroline-1-oxyl (hydrazine mustard spin label—HMSL) was synthesized by the reaction of bis-(2-chloroethyl)-hydrazine with the chloroanhydride of 2,2,5,5-tetramethyl-3-carboxypyroline-1-oxyl mixed in equimolar concentrations in dry tetrahydrofuran at room temperature (the chemical properties of HMSL have been described elsewhere [6]).

(2) 3-[(*N*-Methyl,*N*-chloroethyl)-carbohydrazide]-2,2,5,5-tetramethylpyroline-1-oxyl (methyl chloroethyl-hydrazine spin label—MCEHSL) was synthesized by mixing of 1 mmole of *N*-methyl,*N*-chloroethyl-hydrazine (released from its hydrochloride prior to the reaction) in 5 ml of dry tetrahydrofuran with an equimolar amount of freshly prepared 2,2,5,5-tetramethyl-3-carboxypyroline-1-oxyl chloroanhydride [7]. The reaction mixture was stirred at room temperature for 4 hr, distilled water was added in excess and the reaction product was extracted with ether. The ether layer was washed with water, dried over Na_2SO_4 and evaporated under vacuum. 169 mg (62%) of yellow crystals were obtained from benzene with a m.p. of 124°. The purity of the product was proved by TLC on Kieselgel F_{254} (R_f = 0.52 for a liquid phase of chloroform:methanol = 10:1) and an i.r. spectrum in nujol (3190 cm^{-1}, 1665 cm^{-1}, 1310 cm^{-1}). The alkylating ability of MCEHSL was tested by the reaction of 4-(4-nitrobenzyl)-pyridine [8]. No absorbance at 565 nm for a concentration of 2.7×10^{-6} M was observed.

(3) 3-[*N*,*N*-Bis-(2-bromoethyl)-carbohydrazide]-2,2,5,5-tetramethylpyroline-1-oxyl (bromoethyl-hydrazine spin label—BEHSL) was synthesized by the reaction of 1 mmole of *N*,*N*-bis-(2-bromoethyl)-hydrazine (released prior to the reaction from its hydrochloride) in 5 ml of dry tetrahydrofuran with 1 mmole of freshly prepared 2,2,5,5-tetramethyl-3-carboxypyroline-1-oxyl chloroanhydride [7]. After stirring at room temperature for 3 hr the reaction mixture was extracted with ether. The ether layer was washed consecutively with 0.1 N hydrochloric acid, water, 10% $NaHCO_3$ and water. After drying

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over Na_2SO_4 the ether was evaporated under vacuum. 280 mg (63.3%) of yellow prismatic crystals with a m.p. of 114–115° (melting with decomposition) were obtained from benzene–hexane. Their purity was proved by TLC on Kieselgel F_{254} (R_f = 0.66 for a liquid phase of chloroform : methanol = 10 : 1) and an i.r. spectrum in nujol (3250 cm^{-1}, 1675 cm^{-1}, 1300 cm^{-1}). The alkylating properties of BEHSL were tested by the reaction of 4-(4-nitrobenzyl)-pyridine [8]. The absorbancy at 565 nm for a concentration of 2.0×10^{-6} M in acetone was 0.580.

Rat liver DNA was prepared as described previously [9]. Sedimentation coefficients of the DNA preparations were determined by centrifugation in 1 M NaCl in a Beckman model E analytical ultracentrifuge and the mol. wts were calculated according to Studier [10]. Single-stranded (ss) DNA was prepared by denaturation in 0.01 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) at 98° for 10 min.

Spin-labelling of DNA was carried out by incubation of DNA in 0.01 × SSC at 37° for 24 hr in the presence of an appropriate spin-label at a molar ratio of spin-label to DNA of 1 : 5. The spin-labelled DNA was purified from the unbound spin-label by hydroxyapatite chromatography as follows. The DNA samples were adjusted to 0.03 M phosphate buffer, pH 6.8–0.2 M NaCl and loaded onto water-jacketed hydroxyapatite columns (4–5 ml of bed volume per 1 mg of DNA) pre-equilibrated with the same solution at 50°. The unbound spin-label was removed by 15–20 ml of 0.03 M phosphate buffer, 0.2 M NaCl at 50° and the spin-labelled DNA was eluted with 0.48 M phosphate buffer, pH 6.8, at the same temperature. The 0.48 M phosphate buffer effluents were dialyzed against 0.2 M NaCl and DNA was precipitated with 3 vols of ethanol at −20°.

The spin-labelling of free 5′-deoxyribonucleoside monophosphates (dGMP, dAMP, dCMP and TMP) was carried out by incubating in 0.01 × SSC at a molar ratio of spin-label to dNMP = 1 : 5 at 37° for 24 hr. The spin-label bound to the nucleotides was separated from the unbound one by preparative TLC (Kieselgel F_{254} and chloroform : methanol = 10 : 1 as a mobile phase). The bands corresponding to the alkylated nucleotides and to the free spin-label were collected separately and extracted with 1 N NaOH. The amount of spin-label in both fractions (free and bound) was determined by measuring the area under the EPR spectra of the alkaline solutions.

EPR spectra of the spin-labelled DNAs (with a concentration of 1 mg/ml in 0.1 × SSC) were recorded on an ESR-220 (GDR) spectrometer at room temperature under the following operating conditions: magnetic field 3450 G, power 12.5 mW, modulation amplitude 1 G, scan time 27 min and microwave suppression varying between 12 and 36 dB.

When the amount of spin-label covalently bound to DNA was to be determined the following procedure was employed. EPR spectra of 0.2 mM solutions of the free spin-labels in 0.1 × SSC were recorded, the areas under the spectra were measured and related to 1 mM solutions. Rat liver DNA samples, spin-labelled and purified as described earlier (concentration 1 mg/ml) were digested with DNase I to completion (to make the EPR signal free), EPR spectra of the digests were recorded and the areas under the spectra were measured and related to 1 mM solution of deoxyribonucleotides in DNA. The values obtained were divided by the areas corresponding to 1 mM solutions of the free spin-labels.

The degree of immobilization of the spin-labels as a result of binding to DNA was evaluated by the correlation time (τ_c), the constant $2A_{zz}$ and the ratio K. The correlation time (τ_c) was calculated by the empirical expression:

$$\tau_c = C.\Delta H_0\left[\left(\sqrt{\frac{I_0}{I_+}} + \sqrt{\frac{I_0}{I_-}}\right) - 2\right] \text{ sec.}$$

where I_+, I_0 and I_- are the amplitudes of the low-field, central and high-field resonance lines, and ΔH_0 is the width of the central line in Gauss (G). The constant C for the nitroxyl radicals varies from 5×10^{-10} to 7×10^{-10} G/S. We have employed $C = 6.6 \times 10^{-10}$ G/S. The constant $2A_{zz}$ represents the distance in Gauss between the two extrema of the EPR spectrum. K is a ratio between the intensities of the low- and high-field lines $\left(K = \frac{I_+}{I_-}\right)$.

RESULTS AND DISCUSSION

In a previous paper [6] we have reported the synthesis of a new alkylating spin-label, representing a hydrazine mustard derivative (HMSL) and some preliminary data on its interaction with DNA [11]. The data obtained firmly showed that HMSL was able to alkylate DNA to a sufficient extent to allow a study of the DNA–HMSL complex by the method of EPR. These results encouraged us to widen our study on the interaction of spin-labelled hydrazine mustard derivatives and analogs with DNA aiming to reveal the molecular mechanisms of cytostatic action of this class of anticarcinogens. To this end two analogues of HMSL (MCEHSL and BEHSL) have been originally synthesized in our laboratory. As seen in Fig. 1, MCEHSL has only one chloroethyl group (instead of two, as HMSL) and BEHSL contains two bromoethyl groups (instead of two chloroethyl). These compounds have been chosen for the following reasons: (1) MCEHSL makes it possible to evaluate the significance of the second chloroethyl group for the reactivity of HMSL and the mechanism of its binding to DNA as well, and (2) BEHSL is convenient for studying the role of the halogen nature in alkylating of DNA with hydrazine mustard derivatives.

All three spin-labels showed EPR spectra typical for the nitroxyde radical containing compounds. They consisted of three resonance lines with K = 1.20.

Since it was found that the alkylation of DNA with hydrazine mustard derivatives was salt concentration dependent (results not presented), in all experiments DNA was treated in 0.01 × SSC (corresponding to 0.002 M Na^+) where the reaction rate was maximal. Before recording EPR spectra, the spin-labelled DNA preparations were carefully pur-

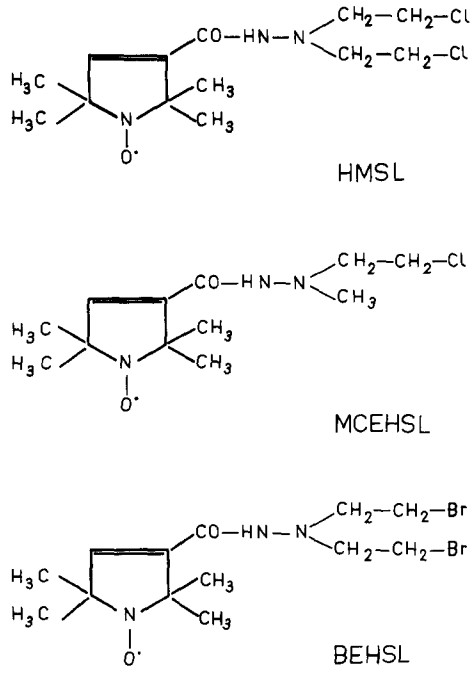

Fig. 1. Chemical formulas of the spin-labelled hydrazine mustard derivatives: HMSL—2,2,5,5-tetramethyl-3-[*N*,*N* - bis - (2-chloroethyl) - carbohydrazide] - pyroline-1-oxyl; MCEHSL—2,2,5,5-tetramethyl-3-[*N*-methyl,*N*-chloroethyl)-carbohydrazide]-pyroline-1-oxyl; and BEHSL—2,2,5,5-tetramethyl-3-[*N*,*N*-bis-(2-bromoethyl)-carbohydrazide]-pyroline-1-oxyl.

Table 1. EPR parameters of double-stranded (ds), single-stranded (ss) and hydrolized DNA spin-labelled with HMSL, MCEHSL and BEHSL

Spin-label	Rat DNA	K	τ_c (sec)	$2A_{zz}$ (G)
HMSL	ds	—	—	44.8
	ss	2.67	9.87×10^{-10}	32.8
	Hydrolized	1.94	—	32.6
MCEHSL	ds	—	—	44.8
	ss	2.65	9.55×10^{-10}	32.8
	Hydrolized	1.94	—	32.6
BEHSL	ds	—	—	34.4
	ss	2.80	1.29×10^{-9}	34.4
	Hydrolized	2.20	—	33.8

K, τ_c and $2A_{zz}$ were determined as described in Materials and Methods.

ified from the unbound spin-label by hydroxyapatite chromatography at 50°. This was the best way (as compared with gel-filtration, ultra-filtration, dialysis and ethanol precipitation) to obtain spin-labelled DNA practically free of unbound spin-label.

In order to study the dependence of the EPR parameters on the secondary structure of DNA both double-stranded (ds) and ssDNA preparations were spin-labelled with the three labelled compounds and their EPR spectra were recorded. As shown in Table 1, the EPR characteristics (K, τ_c, $2A_{zz}$) of the ss spin-labelled DNA are very close for the three

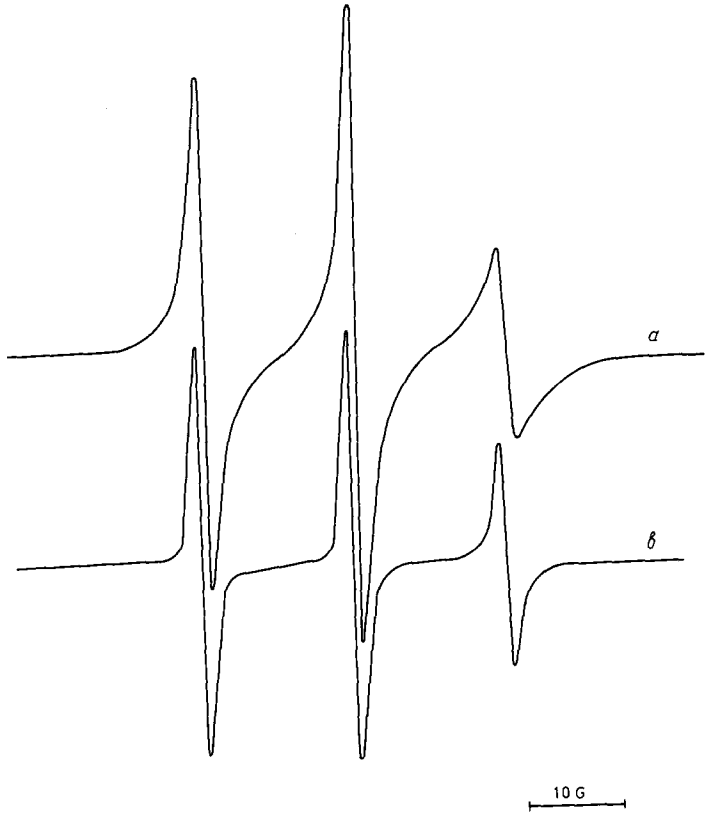

Fig. 2. EPR spectra of single-stranded DNA spin-labelled with HMSL (a) and EPR spectra of hydrolized double-stranded DNA spin-labelled with HMSL (b).

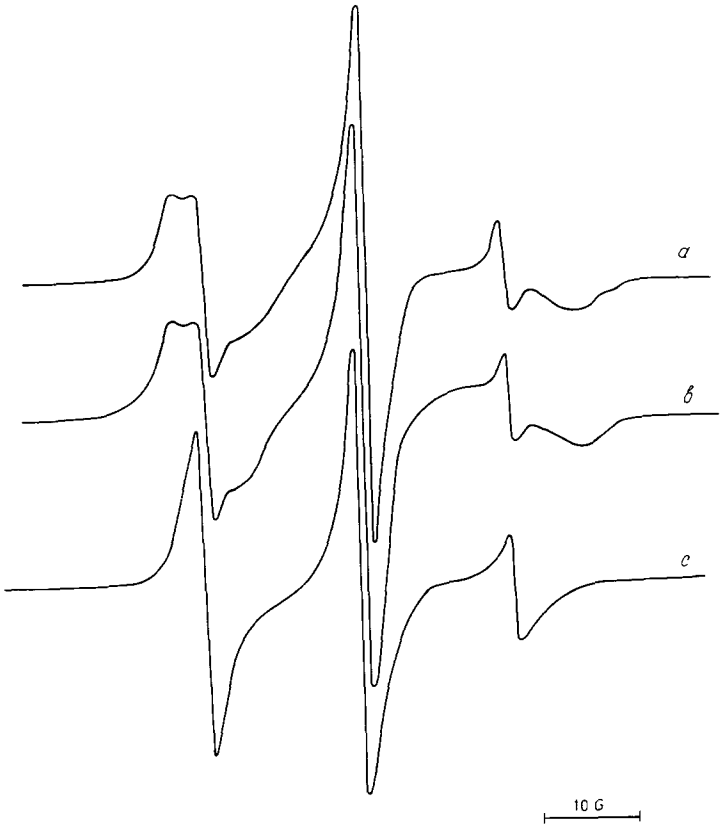

Fig. 3. EPR spectra of double-stranded DNA spin-labelled with HMSL (a), MCEHSL (b) and BEHSL (c).

spin-labels studied (HMSL, MCEHSL and BEHSL) and very similar to those of the hydrolized spin-labelled DNAs. The values obtained characterize the EPR spectra of the ssDNA as non-immobilized. A typical EPR spectrum of such a DNA is presented in Fig. 2.

A quite different picture was observed upon spin-labelling of dsDNA. As seen in Table 1 and Fig. 3 both the EPR parameters and the shape of the EPR spectra for dsDNA spin-labelled with HMSL and MCEHSL are almost identical and very different from the spectrum of the DNA spin-labelled with BEHSL. Whereas the spectra of HMSL- and MCEHSL-treated dsDNA are very immobilized ($2A_{zz}$ = 44.8 G), the spectrum of DNA treated with BEHSL under the same conditions is non-immobilized ($2A_{zz}$ = 34.4 G). As can be seen in Fig. 3, it is very similar to the spectrum of ss spin-labelled DNA.

The identity of the EPR spectra of dsDNA spin-labelled with HMSL and MCEHSL reflects an identical state of both spin-labels in the double helix, i.e. one and the same mode of binding of both substances with DNA. Since MCEHSL is monochloroethyl-containing (see Fig. 1) one can conclude that HMSL reacts with the bases of DNA by only one chloroethyl group. This means that HMSL interacts with only one chain of the double helix and does not form interchain cross-links in DNA. This conclusion is supported by the results on studying the thermal stability of DNA treated with HMSL and MCEHSL (data not shown). It was found that the melting temperature of DNA decreased upon spin-labelling with both HMSL and MCEHSL, i.e. the double helix is labilized rather than stabilized as could be expected if cross-links existed.

The non-immobilized spectrum of DNA treated with BEHSL indicates a high movement freedom of the spin-label bound to DNA. There are two possible explanations of this finding: (1) spin-label molecules might be located in DNA by a way that permits the nitroxyde group (carrying the unpaired electron) to project from the double helix, and (2) BEHSL may destabilize the double helix and generate ss regions in the dsDNA.

To check the second assumption, native DNA preparations were spin-labelled with BEHSL and their secondary structure was studied by hydroxyapatite chromatography and S_1-nuclease treatment (a ss-specific enzyme from *Aspergillus orizae*). It was found that more than 95% of the spin-labelled DNA was eluted from hydroxyapatite with 0.12 M phosphate buffer at room temperature and therefore it represents ss material. This finding was confirmed by the S_1-nuclease assay showing that 96% of the spin-labelled DNA was S_1-nuclease-sensitive.

The results obtained clearly show that, unlike HMSL and MCEHSL, the bromo-analogue of HMSL (BEHSL) has a strong destabilizing effect on DNA. Under the conditions accepted here for

Table 2. Alkylation of dsDNA with HMSL, MCEHSL and BEHSL

Spin-label	mm^2 per 1 mM solution of spin-label	mm^2 per 1 mg/ml DNA	mm^2 per 1 mM solution of deoxynucleotides in DNA	Spin-label bound to DNA (% moles)
HMSL	59,490	994	331	0.55
MCEHSL	59,290	853	284	0.48
BEHSL	67,338	1150	383	0.58

The extent of alkylation of dsDNA with HMSL, MCEHSL and BEHSL was studied as described in Materials and Methods.

spin-labelling of DNA (0.01 × SSC, 24 hr, 37° and spin-label:DNA = 1:5) BEHSL completely denatures DNA. The question arises: What is the reason for the different effects on DNA of these compounds with so similar chemical structures?

One of the possible explanations is to assume that BEHSL alkylates DNA to a greater extent than HMSL owing to its higher reactivity. To check this, the alkylating ability (the amount of spin-label bound to DNA under standard conditions) was studied as described in Materials and Methods and the results are presented in Table 2. As seen in Table 2, the amount of spin-label bound to DNA was almost the same for all three spin-labels tested. These data contradict the assumption mentioned earlier.

Taking into account the equal reactivity of HMSL and its bromo-analogue one can suppose that the different effects of both compounds on the stability of DNA reflects a different mode of binding to the bases of DNA. To ascertain this, the base specificity of the alkylation of DNA with HMSL, MCEHSL and BEHSL was studied. To this end the capability of the four 5′-deoxyribonucleoside monophosphates to bind to every one of the three spin-labels was tested and the results are presented in Table 3. As seen in Table 3, the reactions with HMSL and MCEHSL showed a clear base specificity. They tended to react preferably with the guanilic residues. The binding ability of dGMP was about 3, 7 and 70 times as high as that of dAMP, dCMP and TMP respectively. The affinity of both spin-labels to the four deoxyribonucleotides could be ordered in the following way: dGMP > dAMP > dCMP > TMP. In contrast, the reaction of alkylation with BEHSL did not show any base specificity and this could explain the strong destabilizing effect of this compound on DNA.

Based on the results presented one can speculate that the binding sites for HMSL and MCEHSL are clustered in DNA and most probably they represent GC-rich regions of the DNA molecule. Since these clusters have a higher thermal stability as compared to the AT-rich ones and the latter are affected to a smaller extent upon alkylation with hydrazine mustard chloro-analogues, the stability of the double helix as a whole is preserved. In contrast, the binding sites for BEHSL are randomly distributed in DNA and therefore this compound affects both GC- and the labile AT-rich regions of DNA to an equal extent. Obviously this mode of binding is unfavourable for the stability of the DNA double helix.

The results presented in this study give us reason to draw the following main conclusions:

(1) HMSL and MCEHSL are base-specific alkylating agents reacting preferably with the guanilic residues of DNA. Independently of the different number of chloroethyl groups in their molecules, both compounds have one and the same reactivity with respect to DNA. They alkylate dsDNA giving spin-labelled DNA with immobilized identical EPR spectra and parameters. This indicates that in spite of the presence of a second chloroethyl group in HMSL, it reacts with the bases of DNA by only one of them, i.e. HMSL does not form inter-chain cross-links in the DNA double helix. Spin-labelling of dsDNA with HMSL and MCEHSL is accompanied by a negligible decrease in its thermal stability.

(2) The bromo-analogue of HMSL (BEHSL) is an alkylating agent with no base specificity. Its reactivity to DNA is very close to that of HMSL. The product of alkylation of dsDNA with BEHSL is a spin-labelled DNA with a non-immobilized EPR spectrum similar to that of the ss spin-labelled DNA.

Table 3. Alkylation of 5′-deoxyribonucleoside monophosphates with HMSL, MCEHSL and BEHSL

dNMP alkylated	HMSL bound to dNMP (%)	MCEHSL bound to dNMP (%)	BEHSL bound to dNMP (%)
dGMP	48.6	49.1	24.2
dAMP	18.1	17.7	27.1
dCMP	7.0	6.8	19.3
TMP	0.7	0.7	22.5

The ability of the free 5′-nucleoside monophosphates to bind HMSL, MCEHSL and BEHSL was tested as described in Materials and Methods.

This is due to the fact that BEHSL strongly destabilized and practically denatured dsDNA. Most probably the different effects of HMSL and its bromo-analogue (BEHSL) on DNA are connected with their different base specificities.

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