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Site-directed modification of DNA duplexes by chemical ligation

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ABSTRACT

The efficiency of chemical ligation method have been demonstrated by assembling a number of DNA duplexes with modified sugar phosphate backbone. Condensation on a tetradecanucleotide template of hexa(penta)- and undecanucleotides differing only in the terminal nucleoside residue have been performed using water-soluble carbodiimide as a condensing agent. As was shown by comparing the efficiency of chemical ligation of single-strand breaks in those duplexes, the reaction rate rises 70 or 45 times if the 3'-OH group is substituted with an amino or phosphate group (the yield of products with a phosphoramidate or pyrophosphate bond is 96-100% in 6 d). Changes in the conformation of reacting groups caused by mismatched base pairs (A·A, A·C) as well as the hybrid rU·dA pair or an unpaired base make the template-directed condensation less effective. The thermal stability of DNA duplexes was assayed before and after the chemical ligation. Among all of the modified duplexes, only the duplex containing 3'-rU in the nick was found to be a substrate of T4 DNA ligase.

INTRODUCTION

The efforts of many scientists are focused on studying of synthetic DNA duplexes containing various misincorporations - noncomplementary or inpaired bases, RNA insertion, etc. [1-3]. Investigation of the structure and energetics of such imperfect DNAs is of considerable interest in mechanisms of mutagenesis, repair, recombination, replication as well as hybridization of probes. Site-directed perturbations of the DNA structure allow one to estimate the conformational potentialities of the double helix and to correlate the structure with the duplex stability. Modified DNA duplexes, in particular, with unnatural internucleotide bonds are promising analogs of substrates and inhibitors in studying the mechanism of nucleic acid enzymes. The

increasing demand for modified DNA fragments has stimulated the development of more efficient and precise methods for their synthesis.

One of the possible ways to prepare DNA duplexes containing a single misincorporation is template-directed condensation of modified oligomers using a chemical reagent to activate the phosphate group in a nick (chemical ligation method reviewed in [4]). This method allows one to modify a polynucleotide chain at a particular site when DNA duplexes are being assembled [5,6]. It is not always possible to do their enzymatically because of the substrate specificity of the enzyme. We used this approach before to incorporate phosphoramidate [5] and pyrophosphate [6] internucleotide bonds in DNA duplexes.

In this paper, we employ chemical ligation method for modification of the sugar phosphate backbone of DNA, water-soluble carbodiimide being a coupling agent. To study different modified internucleotide bonds formation the duplexes (I-VIII) with a single nick have been obtained:

(3' —— 5') G-C-C-T-A-G-G-T-C-C-T-C-A-C
(5' —— 3') A-C-G-G-A-X Y-C-A-G-G-A-G T-G-A-C*

I: X = Tp
Y = C
II: X = T
Y = pC
III: X = Tp
Y = 5'-NH_2-C
IV: X = 3'-NH_2-T
Y = pC
V: X = Tp
Y = pC
VI: X = A
Y = pC
VII: X = C
Y = pC
VIII: X = rU
Y = pC

SCHEME 1 * Symbol d is omitted everywhere.

Duplexes I-VIII are composed of a 14-membered oligodeoxyribonucleotide (the template) and of its complementary 6- and 11-membered oligomers which differ only of nucleotide residues facing the nick. The terminal dT or dC of hexa- or undecanucleotides are substituted with 3'(5')-amino- or phosphonucleosides are substituted with 3'(5')-amino- or phosphonucleosides (duplexes III-V); terminal dT of hexanucleotide is replaced either with dA or dC (duplexes VI and VII with non-Watson-Crick dA·dA

and dA·dC b.p.) or with rU (duplex VIII with a hybrid rU·dA pair).

Three more duplexes (IX-XI) were assembled using the same 14-membered template and a pentanucleotide instead of the hexanucleotide while the other oligomer (the undecanucleotide) remained unchanged. These duplexes lack one nucleoside residue at the nick, i.e. one of the template base is unpaired.

(3' —— 5') G-C-C-T-A-G-G-T-C-C-T-C-A-C
(5' —— 3') A-C-G-G-Z Y-C-A-G-G-A-G-T-G-A-C

IX: Z = Ap, Y = C X: Z = A, Y = pC XI: Z = Ap, Y = pC

SCHEME 2

It should be noted that all duplexes studied include the recognition site ($CC^{A}_{T}GG$) of Eco RII restriction endonuclease, the modification being directly adjacent to one of the scissile bonds.

The chemical ligation in duplexes I-XI have been studying to compare the rate and efficiency of formation of natural and modified (pyrophosphate, 5'-N-P or 3'-B-P phosphoramidate) internucleotide bonds, a phosphodiester bond between ribo- and deoxyribonucleotides, phosphodiester bonds changed conformationally by adjacent noncomplementary A·A, A·C base pairs or by an unpaired base and a pyrophosphate bond across a deleted nucleotide residue. Moreover, chemical ligation was used as a specific method to gain information about the conformation of the nicked site. Reacting groups in a DNA duplex are drawn close to one another and oriented owing to forces which stabilize the double helix. A minor perturbation in the structure of the nicked site induced by the above modifications may promptly increase (or decrease) the number of reactive conformers and thus make the chemical ligation more (or less) effective. What makes these findings quite valuable is the recently discovered phenomenon of self-splicing, namely, in vivo site-directed breakdown and rejoining of bonds in the RNA backbone without an enzyme being involved in this process [7]. The above nick-containing duplexes were used to compare the effectiveness of

water-soluble carbodiimide induced ligation versus enzymatic one.

MATERIALS AND METHODS

General. Used in the work were CDI, MES [8], Lichrosorb NH_2 resin (5 μm) (Merck), acrylamide, N,N'-methylenbisacrylamide, γ-^{32}P-ATP (Amersham), T4 DNA ligase and RNase A (USSR), bacterial alkaline phosphatase (Sigma).

The following buffer solutions were used: buffer 1 (for chemical ligation) - 50 mM MES, pH 6.0, 20 mM $MgCl_2$; buffer II - (for T4 DNA ligase) - 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol; buffer III (for melting ligated duplexes) 0.015 M citrate Na, 0.15 M NaCl, pH 7.2.

Oligodeoxyribonucleotides were synthesized by the phosphotriester method as described previously [9]. $ACGGAT_{NH_2}$ and NH_2CCAGGAGTGAC were prepared analogously, using corresponding azidonucleosides as aminonucleoside precursors. ACGGATp was obtained from ACGGATrU by peridate oxidation of the 2',3'-cis-hydroxyl system followed by β-elimination [10]. ACGGAp was obtained from ACGGArU in the same way. ACGGA was prepared by ACGGAp dephosphorylation using alkaline phosphatase.

5'-^{32}P-phosphorylation of oligonucleotides and electrophoresis in polyacrylamide gel were effected by standard procedures [11]. MCC was carried out by using "Milichrom" (USSR) equipped with a Lichrosorb-NH_2 column (1x30 mm, 5 μm) in a sodium-phosphate linear gradient (0-0.3 M), pH 7.0 in 7 M urea.

Optical Measurement. The temperature dependences of UV absorption of the oligonucleotide mixtures were measured on Cary-219 (USA) spectrophotometer in thermostatted quartz cells of 1 mm path length. Equimolar component mixtures were used with the total strand concentration C_o = (0.2-0.5)x10^{-4}M in buffer I or III. The transition enthalpy ($\Delta H°$) and melting temperature (T_m) were determined from the differential melting curves using equations [12]. The hypochromicity (h) was calculated from the melting profiles. CD spectra were measured on a Roussel-Jouan III dichrograph (France) at concentration of (0.2-0.5)x10^{-4}M, buffer I or III, 20°C.

Chemical ligation (general method). A equimolar mixtures of oligonucleotides in 20 µl of buffer I was cooled to 0°C and then 0.03 g CDI was added. In the reaction mixture the final concentration of oligomers was 0.1 mM, CDI - 0.2 M. The mixture was incubated in the dark at 0°C. The time course of the reaction varied depending on its effectiveness. After incubation the oligonucleotide fraction was precipitated with ethanol, dried and analyzed by MCC or by electrophoresis in polyacrylamide gel. In the last case to reaction mixture was added 5'-^{32}P--labeled undecanucleotide ($\sim$ 10-15 nCi ^{32}P). Ligation products were isolated by MCC.

Enzymatic ligation was carried out in 10 µl of buffer II, which contained 0.01 mM hexa(penta)nucleotide, 0.01 mM tetradecanucleotide and 0.01 mM 5'-phosphorylated undecanucleotide containing ^{32}P-label ($\sim$ 10 nCi), 1 mM ATP and 5 units of T4 DNA ligase. The mixtures were incubated for 12 hr at 8°C. The reaction was terminated by addition of EDTA to 20 mM concentration and electrophoresed in polyacrylamide gel.

Selective cleavage of a phosphoamide bond. 0.2 A_{260} of the ligated duplex III' or IV' was dissolved in 10 µl of 15% CH_3COOH and kept in a water bath for 5 min at 95°C. The solution was evaporated *in vacuo* and last traces of acetic acid were completely removed by repeated coevaporation with ethanol. Products were analyzed by MCC.

Selective cleavage of a pyrophosphate bond [6]. 0.2 A_{260} of ligated duplexes V' or XI' was dissolved in 15 µl of pyridine-water mixture (9:1) and treated with 10 µl of freshly distilled $(CF_3CO)_2O$. The oligonucleotide fraction was precipitated with ethanol, dried and analyzed by MCC.

Cleavage of a phosphodiester bond between ribo- and deoxyribonucleotide residues. To 0.01 A_{260} ^{32}P-labeled ACGGArU*pCCAGGATGAC in 5 µl of 0.1 M Tris-HCl, pH 7.5, was added 5 µl of the RNase A solution (1 mg/ml) in the same buffer. The mixture was kept at 37°C for 1 hr. The oligonucleotide fraction was precipitated with alcohol and electrophoresed in polyacrylamide gel.

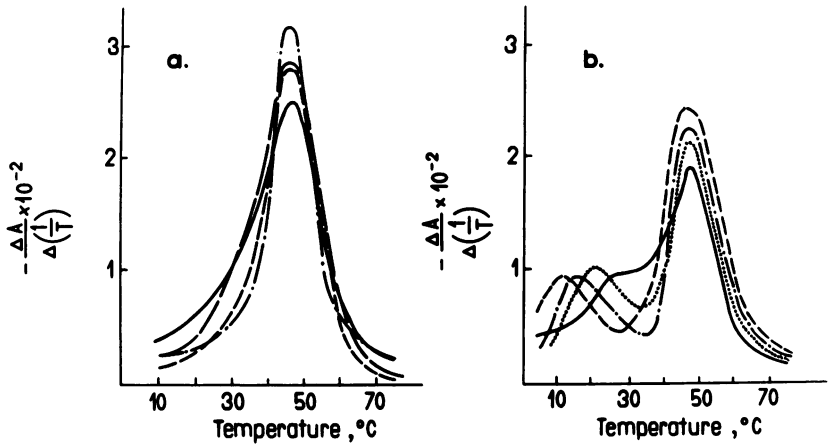


Figure 1. Differential melting curves for nick-containing duplexes: a) (I) — —, (IV) — — —, (V) ————, (XII) — · — · —, b) (VI) ····· , (VII) — · — , (VIII) ————, (IX) — — — —. For duplex composition see Schemes I and II. C_o $(0.2\text{-}0.4)\cdot 10^{-4}$M, buffer I.

RESULTS

Studying the thermodynamic properties of duplexes I-XI would provide important information for chemical ligation experiment, because the efficiency of coupling reaction is correlated with the stability of double-stranded DNAs [4]. There is another reason for such studies. Although the effect of a misincorporation on the structure and properties of mini-duplexes was reported [1,12-15] hardly any work has been done with DNA duplexes containing a nick in the modified site.

The melting characteristics of nick-containing duplexes I-XI were investigated using their UV absorbance-temperature profiles. The values of h, T_m and $\Delta H°$, are listed in Table I. The table shows also these values for duplex XII composed of a 14-mer template and complementary undecanucleotide. Fig. 1 presents differential melting curves for some of the duplexes. Only normally-paired DNA duplexes I-V have a monophasic thermal profile (Fig. 1a). Duplexes VI-XI exhibit a biphasic melting which is indicative of two structural transitions (Fig. 1b). The first one at a low temperature (Table I) is almost certainly a separation of a modified hexa(penta)nucleotide forming

TABLE I. Thermodynamic data for nick-containing duplexes (buffer I, C_o $0.3 \cdot 10^{-4}$M)

Duplex number	T_m, °C ± 1		$\Delta H°$, kcal/mol ± 3		h, % ± 0.5
I	47.0		55		18.5
II	47.0		58		19.0
III	46.0		82		20.0
IV	47.0		82		19.0
V	47.0		57		18.5
	Trans I	Trans II	Trans I	Trans II	
VI	18.0	47.0	55	70	17.5
VII	15.0	49.0	37	67	16.0
VIII	26.5	48.0	-	-	17.5
IX	10.5	47.5	42	65	16.0
XII*	-	47.0	-	70	17.0

* (3'-5') GCCTAGGTCCTCAC
(5'-3') CCAGGAGTGAC

either a mismatched base pair or a hybrid base pair or a hybrid base pair or a nonpaired base). Its demitransition temperature (T_{Im}) increases in the order: extra adenosine residue, dA·dC base pair, dA·dA base pair, hybrid rU·dA base pair. The results are in agreement with what should be expected from [1,14,16]. The second transition corresponds to the melting of the duplex composed of a template and of an undecanucleotide into the single-stranded state, and its semitransition temperature (T_{IIm}) correlates quite well with the T_m of control duplex XII (Table I). The enthalpic changes involved in both transitions were calculated by analysing the shape of differential melting curves for duplexes VI-IX (Table I). The values of $\Delta H°$ are calculated for each peak separately. The overall $\Delta H°$ values were found to be almost the same as those for ligated duplexes VI'-XI' (Table 2). For duplexes I-V having monophasic melting curves, $\Delta H°$ values are lamost twice as low as the ones for the corresponding ligated duplexes (cf. Tables I and 2). Presumably, in duplexes I-V, hexa- and undecanucleotides separate from the

TABLE 2. Thermodynamic data for ligated duplexes (buffer III; C_o $0.3 \cdot 10^{-4}$M)

Duplex number*	Duplex composition**	T_m, °C±1	$\Delta H°$, kcal/mol±3
I',II'	3' G-C-C-T-A-G-G-T-C-C-T-C-A-C 5' A-C-G-G-A-T-C-C-A-G-G-A-G-T-G-A-C	62	136
IV'	T-A - G A-T_{Np} C	63	125
V'	T-A - G A-T_{pp} C	62	124
VI'	T-A-G A-A-C	55	119
VII'	T-A-G A-C-C	52	118
VIII'	T -A-G A-rU-C	60	122
IX',X'	T-A-G A - C	52	104
XI'	T-A-G Ap pC	52	104

* The ligated duplexes have the same number but with touch as the corresponding nick-containing ones.

** The mismatches and other anomalies are indicated in quadrangles below perfect duplexes.

complementary template in two steps also, but the temperature interval between two transitions is small. Here the $\Delta H°$ value calculated from the differential melting curve, which is a super-position of two closely located curves, will inevitably be understated. The existence of "strong" and "weak" sites in such duplexes stems from the heterogeneous length and composition of oligomers bound to the template and, strictly speaking, conformational transitions in such duplexes cannot be described in terms of the "all-or-none" model. Obviously, interoligomeric stabilization would make the melting of duplexes more coopera-

tive. Indeed, the $\Delta H°$ values for the formation of duplexes III and IV containing oligonucleotides with a terminal amino groups are much higher than those for duplexes I, II and V. This is determined by an additional electrostatic interaction between the amino and phosphate groups of adjacent oligonucleotides.

The results of our experiments have shown that, under the conditions used, duplexes I-XI are stable at a temperature below 5°C and are a suitable model for studying the template-directed chemical reactions.

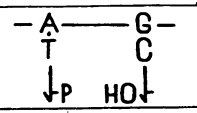
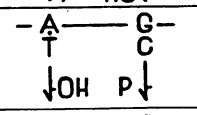
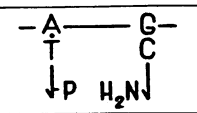
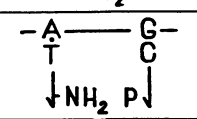
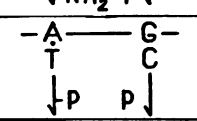
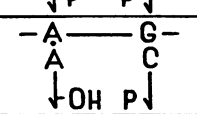
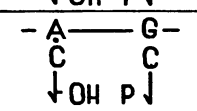
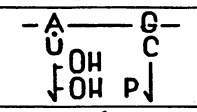
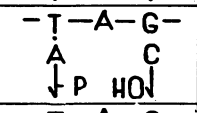
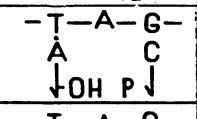
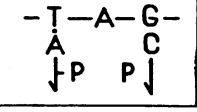
Chemical ligation. CDI was used as a condensing agent in aqueous solution. To optimize the conditions of the coupling reaction, we used duplex I (Scheme I) composed of an undecanucleotide and a non-modified hexanucleotide with a 3'-terminal phosphate group. In this duplex, the 3'-phosphate is attacked by the reactive 5'-hydroxy group in the adjacent oligomer. No condensation of hexa- and undecanucleotides was shown to take place in the absence of template-tetradecanucleotide proving that the coupling reaction was, indeed, template-directed.

The concentration of CDI and oligonucleotides, the temperature and the pH of buffer I were varied. All the reaction mixtures were analysed by MCC after 24 h.

When CDI concentration was raised from 0.05 to 0.2 M, the yield of the ligation product increased from 48 to 70%, CDI-modification products being virtually absent from the reaction mixtures. A further rise of CDI concentration did not affect the heptadecanucleotide yield.

A 10-fold decrease in oligonucleotide concentration of 10^{-4}M makes the product yield fall from 70 to 40%, presumably because the duplex was partly destroyed. The coupling reaction was studied from -15° to 37°C and the product yield was highest (70%) at 0-10°C. The chemical ligation was retarded (yield 37%) in a frozen solution and the heptadecanucleotide yield was reduced at a temperature above 10°C since more of the duplex melted. Moreover, by-products of oligonucleotide modification with CDI [17] accumulated in the reaction mixture as the temperature was elevated. The reaction rate hardly changed and non by-products were accumulated when the pH 6 of the buffer was changed to either 5 to 7.

TABLE 3. The Results of Chemical and Enzymatic Ligation of Nicks in duplexes I-IX*

Duplex number	Reactive site structure	Ligation by CDI**		Ligation by T4 DNA ligase** (yield, %)
		Optimal coupling time, days	Yield, %	
I		4	95	0
II		6	75	95
III		0.25	97	0
IV		0.25	97	0
V		0.25	92	0
VI		6	32	0
VII		6	50	0
VIII		6	28	80
IX		6	25	0
X		6	8-10	0
XI		4	85	0

* Duplex composition see Scheme 1 and 2
** For conditions, see "Materials and Methods"

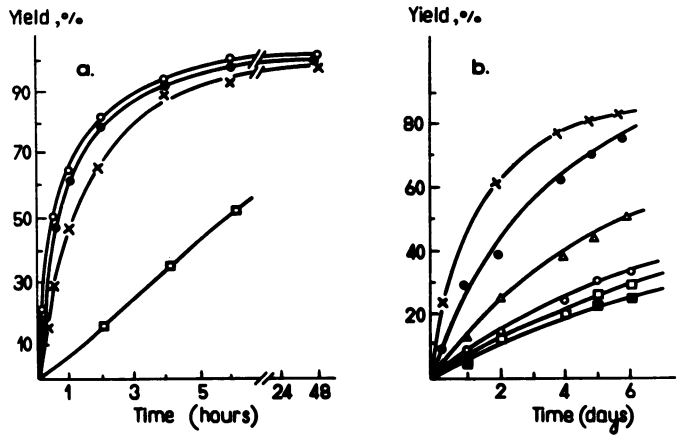


Figure 2. Accumulation of nick-ligation products in duplexes: a) (I)—□—, (III) —○—, (IV) —●—, (V) —×—, b) (ii)—●—, (VI)—○—, (VII)—△—, (VIII)—□—, (IX)—■—, (XI)—×—. For duplex composition see Scheme 1, for condition of CDI-induced coupling reaction, see Materials and Methods.

The carbodiimide coupling of oligonucleotides into duplexes I-XI (Table 3) under the selected optimal conditions (C_o 10^{-4}M; 0.2 M CDI; 0-5°C; buffer I, pH 6.0) was studied. The reaction mixtures containing duplexes I, III and IX with a 3'-phosphorylated hexa(penta)nucleotide were analysed by MCC. The mixtures containing duplexes II, IV-VIII, X and XI with a 5'-phosphorylated undecanucleotide were analysed by polyacrylamide gel electrophoresis. Chemical ligation was found to occur in all the mixtures, but its rate and efficiency were not identical. For the convenience of analysis, the duplexes were subdivided into 2 groups.

The first group included duplexes I-V in which a natural phosphodiester, phosphoramidate or pyrophosphate bond is formed (Table 3). Accumulation of chemical ligation products in duplexes I-V is presented in Fig. 2a and autoradiograms of the same reaction mixtures are in Fig. 3. One can see that the rate of the coupling reaction depends strongly on the nature of a group that attacks the activated phosphate. This agrees well with the data reported earlier for the concatemeric duplexes [4-6]. The reaction rate rises 70 or 45 times if the 3'-OH group in

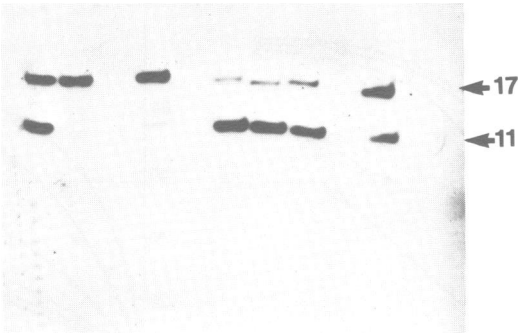


Figure 3. Polyacrylamide gel electrophoresis analysis of reaction mixtures after CDI-induced nick ligation in duplexes II, IV-VIII, XI for 2 days. For condition see Material and Methods. Lane numbers correspond to duplex numbers (Table 3). The chain length of oligonucleotide are indicated to the right. XC and BPB, xylene cyanol and bromphenon blue markers respectively.

the coupling site is substituted with a more nucleophilic amino or phosphate group, respectively. The yield of a heptadecanucleotide with a pyrophosphate or phosphoramidate internucleotide bond approximates 100% within 6 h and the phosphoramidate bond is formed at a higher rate than the pyrophosphate bond.

As follows from comparing the rates of the coupling reaction in duplexes I-IV (Fig. 2a), an internucleotide bond is formed more readily if either a reacting hydroxy (duplex I) or an amino group (duplex III) are at the 5'-termini of the oligomer. The rate of phosphodiester bond formation in duplex I is 10 times as high as that in duplex II. However, these rates differ only 1.4 times when a phosphoramidate bond is formed (Fig. 2a, duplexes III and IV). Apparently, the higher nucleophilicity, the less is the difference in the reactivity of groups differing in position (5'-or 3'-) in the coupling site.

The second group included duplexes VI-XI with considerable structural perturbations at a single-strand break (Table 3). With the exception of duplex XI, the rate of chemical ligation in these duplexes was much lower as compared to that of the first group (Fig. 2b).

The efficiency of coupling reaction was least in duplexes IX and X containing an unpaired base near the single break

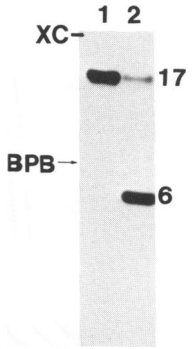


Figure 4. Polyacrylamide gel electrophoretic analysis of ACGGArUpCCAGGAGTGAC before (1) and after (2) cleavage by RNAse A. The position of ^{32}P-label is denoted by asterisk. Symbols are indicated in legend to Fig 3.

(yields 25 and 8-10% respectively). This may be due to the insufficient proximity of the reacting groups. An introduction of a second phosphate group in a nick (Table 3, duplex XI) results in a partial closing of the gap and moreover, in a substitution of the hydroxy group with a more nucleophilic phosphate group. As a result, the rate of pyrophosphate linkage formation in duplex XI is ten times greater than in duplex IX and even exceeds the rate of phosphodiester linkage formation in normally-paired duplex II (Fig. 2b).

The low efficiency of nick ligation in mismatched duplexes VI and VII (coupling yields 32 and 50% respectively) was caused by the fact that A·A and A·C base pairs localized at the nick distort the optimal orientation of reacting groups. This is to be expected since incorporation of noncomplementary base pairs in the interior of the double helix is known to result in a local perturbation of the sugar phosphate backbone [15,18].

The data on the chemical ligation in duplex VIII containing a hybrid rU·dA pair (Table 3) are noteworthy. As can be seen in Fig. 2b, heptadecanucleotide yield is only ca. 30%. Presumably, a fixed unfavourable conformation of the reactiving groups in this duplex is what makes the chemical ligation ineffective. This supposition is based on studying the structure of duplexes composed of covalently bonded RNA and DNA fragments

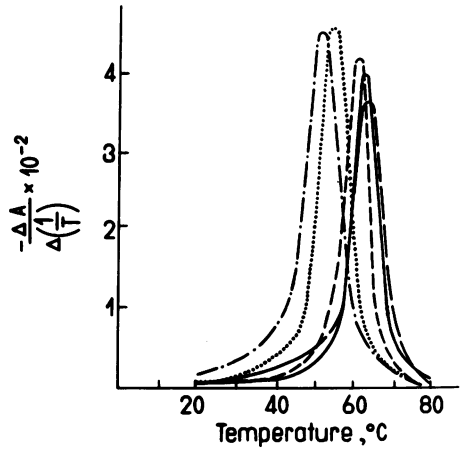


Figure 5. Differential melting curves of the ligated duplexes: (I')——— , (IV')— — , (VI')·····, (VII')—·—, (VIII')— — —. For duplex composition see Table 2. C_o $0.2 \cdot 10^{-4}$M, buffer III.

[3,19,20].

The synthesized internucleotide linkage in ACGGArUCCAGGAGT-GAC was characterized by RNAse A digestion. The phosphodiester linkage between rU and dC was hydrolyzed with the enzyme by 86% (Fig. 4). Thus, 3'-5' linked oligomers were formed predominantly in duplex VIII. Such a regiospecifity of the coupling reaction seems to be due to the duplex structure.

The primary structure of oligonucleotides prepared by the nick ligation in duplexes I, II, VI, VII, IX and X was confirmed by the Maxam-Gilbert analysis. The nature of the resultant unnatural phosphoramidate and pyrophosphate inter-oligomer bonds was proved by selective cleavage of chemical ligation products with acetic acid (duplexes III' and IV') or by trifluoroacetic anhydride (duplexes V' and XI'). In all cases, the cleavage yielded the starting oligonucleotides.

The structure of the chemical ligation products was also corroborated by analysing their melting behavior. The differetial melting profiles of some ligated duplexes differing by a single structural alteration are shown in Fig. 5 and their T_m and $\Delta H°$ values are listed in Table 2. The ligated duplexes shown higher T_m values when compared to the nick-containing

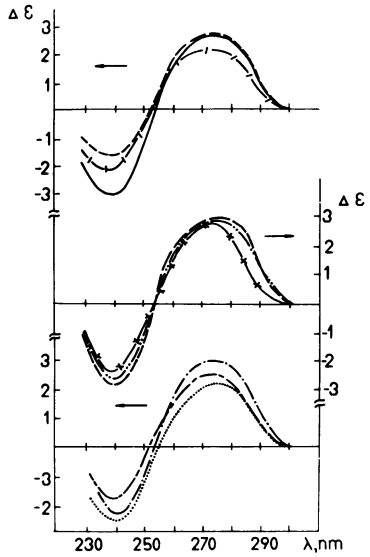


Figure 6. CD spectra of the ligated duplexes: (I')——— , (IV') — — , (V')—··—, (VI')••••••• , (VII')—·—·—, (VIII') — — — , (IX')—— — —, (XI')—×— . For comparison the CD spectrum of the duplex I (—/—) before chemical ligation is given. For duplex composition see Table 2. C_o $0.2 \cdot 10^{-4}$M, buffer III.

duplexes (cf. Table 1 and 2). Moreover, all the investigated ligated duplexes have monophasic melting curves, while some nick-containing duplexes exhibit a biphasic thermal profile. A specific modification (mismatched or inserted bases or a hybrid base pair) reduces the T_m values of ligated duplexes (Table 2). The order of destabilization agrees well with that for the corresponding nick-containing duplexes. The replacement of one phosphodiester bond with a pyrophosphate or phosphoramidate bond hardly changes the melting temperature of the obtained 14-mer DNA duplexes (Table 2). The enthalpy changes for the formation of imperfect duplexes IV'-IX' and XI' are somewhat lower than those for nonmodified duplex I' (Table 2). These data are consistent with the published ones [1,12].

The CD spectra of the ligated duplexes (Fig. 6) have a conservative shape with the positive Cotton effect at 260 nm which is characteristic of the B-type conformation. The spectra of

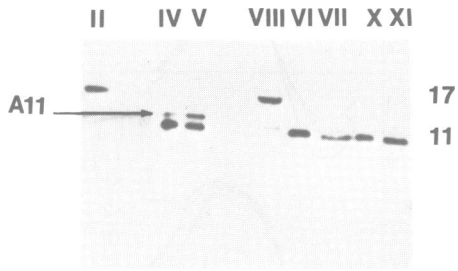


Figure 7. Polyacrylamide gel electrophoretic analysis of reaction mixtures containing duplexes II, IV-VIII, X, XI after incubation with T4 DNA ligase at 8-10°C for 12-15 hrs. A11- proposed undecanucleotide-adenylate [21]. For conditions and details, see text.

all ligated duplexes are similar to one another, thus testifying to the identical geometry of double helices. Hence, the conformational changes induced by the modification are localized in the helix. The CD spectra were shown to have no drastic differences in the shape for the ligated and nick-containing duplexes (Fig. 6).

Enzymatic ligation. Duplexes II, IV-VIII, X and XI were subjected to the action of T4 DNA ligase under standard conditions. Apart from control duplex II, the coupling reaction was effected only in duplex VIII containing an rU and pdC in the nick (Fig. 7 and Table 3). The product yield was 80% and only a 3'-5'-phosphodiester linkage was formed between rU and dC (hydrolysis with RNAse A). Effective ligation in duplex VIII was to be expected because T4 DNA ligase joins ribo- and deoxyribonucleotide blocks on a DNA template [20].

T4 DNA ligase cannot sealing of nicks in duplexes IV and V (Table 3) whose 3'-terminal hydroxy group is replaced with an amino or a phosphate group. Apparently, the enzyme-substrate complex formed is nonproductive due to the alien chemical nature of the nucleophile. T4 DNA ligase was also found to be incapable of catalyzing the formation of usual phosphodiester bonds when the structure of the reaction site was deformated

for instance, by noncomplementary base pairs (duplexes VI and VII) or by deleting a nucleoside residue (duplex X).

Our results on the latter group of complexes disagree with the published data that T4 DNA ligase catalyzes the joining of hydrogen-bonded duplexes containing 3'-end - mismatched nucleotides [22,23], as well as gapped circular DNA molecules [24]. Apparently, the substrate specificity of the enzyme can be extended under certain conditions. We made our enzymatic ligation experiments under identical conditions to compare correctly the results for a series of DNA duplexes differing only in the structure of a ligation site. These data are of interest for studying the active site of DNA ligase and its substrate specificity.

Conclusion. The results described in this paper show that chemical ligation is a very promising technique for obtaining DNA and DNA-RNA hybrid duplexes with various sugar phosphate backbone modifications. Enzymatic ligation was found to be ineffective for all the investigated duplexes, with the exception of duplex VIII containing rU in the coupling site. Substitution of a more nucleophilic phosphate or amino groups for the hydroxy group makes chemical ligation more effective, and the yield of products approaches 100% after 6 d. A change in the orientation and proximity of the reacting groups, caused by a mismatched base pairs or an unpaired base, decreases the coupling yield as compared to the perfect duplex. The reaction is most favoured when the hydroxy and phosphate groups are in a conformation corresponding to the DNA B-type. If this structure is violated, the chemical ligation decelerates and its effectiveness indicates how much the conformation of a modified site differs from the canonical one. The template-directed coupling of ribo- and deoxyribonucleotide residues under the action of the chemical agent relatively inefficient but regiospecific, so that a 3'-5' phosphodiester linkage is predominantly synthesized.

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8. Abbreviations: CDI, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide, hydrochloride; MES, 2-morpholinoethanesulfonate; T_{NH_2}, 3'-amino-3'-deoxythymidine; $ACGGAT_{NH_2}$, hexanucleotide containing T_{NH_2}; $_{NH_2}C$, 5'-amino-5'-deoxycytidine, $_{NH_2}CCAGG$-AGTGAC, undecanucleotide containing $_{NH_2}C$; ... $N_{NP}N$ and ...NppN..., oligomers containing (3'-5')phosphoramidate and pyrophosphate bonds respectively; MCC, microcolumn chromatography.
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