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Brief Communication

Efficient Synthesis of DNA Dumbbells Using Template-Induced Chemical Ligation in Double-Stranded Polynucleotides Closed by Minihairpin Fragments

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ABSTRACT

The chemical ligation of 17 50–54-membered nicked DNA dumbbells with different closing fragments, nick positions, and nucleotides facing the nick were investigated. T_4, T_5, GTA_4C, $GCGA_2GC$, and $GCGA_3GC$ sequences were chosen as the closing fragments. The nicks were placed in the center of the duplex stem or were adjacent to the closing fragments. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide and cyanogen bromide were used as the condensing agents. We showed that the ligation efficiency is 10%–90% depending on the sequence of the closing fragments, nick position, and nucleotides facing the nick. Coupling yields of 80%–90% were observed when the nick was situated in the middle of the molecule between two T residues or was adjacent to $GCGA_2GC$ or $GCGA_3GC$ minihairpins. In the last case, the reacting 3′-phosphate and 5′-hydroxy groups were brought close together by only two base pair minihairpins. The coupling yields did not depend on the nature of the condensing agent. On the basis of the results obtained, we believe a rational design of nicked DNA dumbbells has been developed for efficient chemical synthesis of closed dumbbells.

INTRODUCTION

DNA DUPLEXES COVALENTLY CLOSED on both ends by nucleotidic loops or synthetic linkers (DNA dumbbells) are widely used for structural investigation of nucleic acids (Doktycz et al., 1992, 1993; Amaratunga et al., 1992; Paner et al., 1992; Ippel et al., 1995a,b). They provide clear advantages over linear DNA because of the absence of the conformational heterogeneity caused by monomer-dimer equilibrium and the effect of fraying ends. Studies of dumbbells have permitted an unambiguous comparison of optical melting and temperature-induced exchange broadening of the NMR spectrum of DNA (Benight et al., 1988). The usefulness of DNA dumbbells has been demonstrated in the calculation of the relative contributions of the stacking interaction of each pair, which can be used to predict the overall stability of a DNA molecule from its sequence (Doktycz et al., 1992). DNA dumbbells have been used to investigate the thermodynamics of intramolecular loop formation (Amaratunga et al., 1992; Paner et al., 1992; Doktycz et al., 1993; Ippel et al., 1995a,b). In addition, DNA dumbbells were found to be far better substrates for studies of triplex stability than linear duplexes (Paner et al., 1993).

The sense approach for specific regulation of gene expression at the transcription level has been proposed (Riabowol et al., 1992; Clusel et al., 1993; Bielinska et al., 1990; Tanaka et al., 1994; Ma et al., 1993). This approach is based on the use of ds oligonucleotides containing sequences recognized by transcription factors as decoys for these factors. DNA dumbbells were found to be more advantageous as compared with dsDNA, as their sequences cannot undergo strand separation. In addition, they possess increased stability toward nucleolytic degradation because of the absence of free termini (Clusel et al., 1993; Chu

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and Orgel, 1992). It has been reported that transcription factors that bind to standard dsDNA bind with equal or greater affinity to equivalent DNA dumbbells (Clusel et al., 1993; Chu and Orgel, 1991). Also, we have recently demonstrated that stabilization of ds sequences, formed by connecting both ends with nucleotidic or synthetic loops (dumbbell structure), permits detection of efficient, specific inhibition of gene expression *in vitro* and *ex vivo* at nanomolar concentration. However, under the same conditions, the ds oligonucleotides were inactive (Clusel et al., 1993; I.N. Merenkova et al., unpublished observations). Dumbbell decoys, by controlling the expression of disease-causing genes, may find uses in medicine. Therefore, they are attractive tools for both research and applied biotechnology.

Several methods for the synthesis of DNA dumbbells have been investigated (Ashley and Kushlan, 1991; Barbato et al., 1989; Lim and Hunt, 1997; Wemmer and Benight, 1985; Erie et al., 1989). Enzymatic ligation of nicked dumbbells or sticky-ended hairpins has been applied successfully to obtain DNA dumbbells (Doktycz et al., 1993; Clusel et al., 1993; Wemmer and Benight, 1985; Erie et al., 1989). However, this approach is limited in scope by the expense of DNA ligase and is undesirable for large-scale production. Besides that, ligation of two sticky-ended hairpins cannot be used to generate small dumbbells (Wemmer and Benight, 1985). It has been reported that the specificity of the ligase may severely constrain the size of the dumbbells that can be prepared (Erie et al., 1989).

Another method, based on closing the noncircular fragments via a solid-phase nontemplate approach, has been developed (Barbato et al., 1989). This approach has yielded cyclic DNA only for short oligomers ($\leq$14nt in length) because nontemplate (random) circularization is a slow, entropically unfavorable process for the formation of large rings (Rubin et al., 1995).

Template-induced chemical ligation, using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) as a condensing agent, has been proposed to obtain DNA dumbbells in multimicromolar quantities (Ashley and Kushlan, 1991). However, a lengthy reaction time (16–96 hours) (Ashley and Kushlan, 1991; Kuznetsova et al., 1996a,b), the propensity of EDC to covalently modify unpaired bases, and the absence of data about rational design of the nicked precursors limit the use of chemical ligation.

In the present work, we have developed a chemical ligation method for obtaining unmodified phosphodiester DNA dumbbells. We have proposed using cyanogen bromide as an efficient condensing agent for DNA dumbbell synthesis. A set of 17 50–54-membered nicked DNA dumbbells containing the binding site of HNF1 transcription factor was used in this work. We have investigated the dependence of the chemical ligation efficiency on (1) the nick position, (2) the nature of nucleotides in the nick, (3) the structure and stability of the closing loops, and (4) the nature of the condensing agent. Here, we present a rapid, efficient, and purely chemical technique for synthesis of DNA dumbbells based on a rational design of nicked precursors.

MATERIALS AND METHODS

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized by GENSET (Paris, France) using an automated DNA synthesizer (Applied Biosystems 394/8, Foster City, CA). All initial oligonucleotides were 3′-phosphorylated. The 3′-terminal phosphate group (3′-p) was introduced into the oligonucleotides as previously described (Purmal et al., 1992).

The structures and sequences of the oligonucleotides used in this study are depicted in Figure 1. All oligonucleotides form nicked DNA dumbbells when 3′-phosphate and 5′-hydroxy groups are adjacent to the nicks.

Synthesis of DNA dumbbells by EDC-induced chemical ligation

Synthesis of DNA dumbbells using EDC as a condensing agent was performed as follows. Initial nicked DNA dumbbells were dissolved in 0.05 M MES (2-(N-morpholino)ethane sulfonic acid), pH 6.0, 0.02 M $MgCl_2$ (buffer A) to an oligonucleotide concentration of 10^{-3} M (per monomer). Reaction mixtures were incubated at 90°C for 10 minutes and slowly cooled to 0°C in 2–3 hours. Then, 2 M EDC in buffer A was added (1/10 of the total volume). After 12–16 hours at 0°C, the reaction mixtures were precipitated with 10 volumes of 2% $LiClO_4$ in acetone, dried, and analyzed by electrophoresis on a 12% denaturing polyacrylamide gel (PAGE) without a rapid heat-cool step before loading. EtBr staining was used to visualize the DNA bands. The ligation efficiency was determined by gel scanning with BPMA-Shater microcomputer equipment complex (Deltathekh Ltd., Moscow, Russia).

Synthesis of DNA dumbbells by cyanogen bromide (CNBr)-induced chemical ligation

Nicked DNA dumbbells were dissolved in 0.25 M MES adjusted with $(C_2H_5)_3N$ to pH 7.5, 0.02 M $MgCl_2$ (buffer B) to an oligonucleotide concentration of 10^{-3} M (per monomer). After annealing, 5 M CNBr in dry acetonitrile was added (1/10 of the total volume). After 2 minutes at 0°C, the reaction mixtures were precipitated with ethanol, dried, and analyzed as described.

RESULTS

Design of nicked DNA dumbbells

As has been reported previously for linear DNA duplexes, the efficiency of chemical ligation strongly depends on the following factors: (1) the stability of the duplex to be ligated, (2) the localization of the phosphate group participating in the ligation (on the 3′-end or 5′-end of the oligomer), (3) the nature of the nucleotide residues adjacent to the nick, and (4) the nature of the condensing agent (Shabarova, 1988; Dolinnaya et al., 1991; Merenkova et al., 1992). In addition, the structure and sequence of the closing loops have to be considered for successful chemical ligation of nicked dumbbells. Taking into account these factors, we developed the following design for nicked DNA dumbbells (Fig. 1). All DNA dumbbells contain the 15 bp binding site of the HNF1 transcription factor. As the closing fragments, the hepta and octamer sequences 5′-GCGA_2GC-3′ and 5′-GCGA_3GC-3′ were chosen (nicked DNA dumbbells III_{1-5} and IV_{1-5}). The extraordinary stability of these minihairpin structures (T_m 76.5°C) (Yoshizawa et al., 1994) was shown previously. These fragments are stabilized by two G-C and one

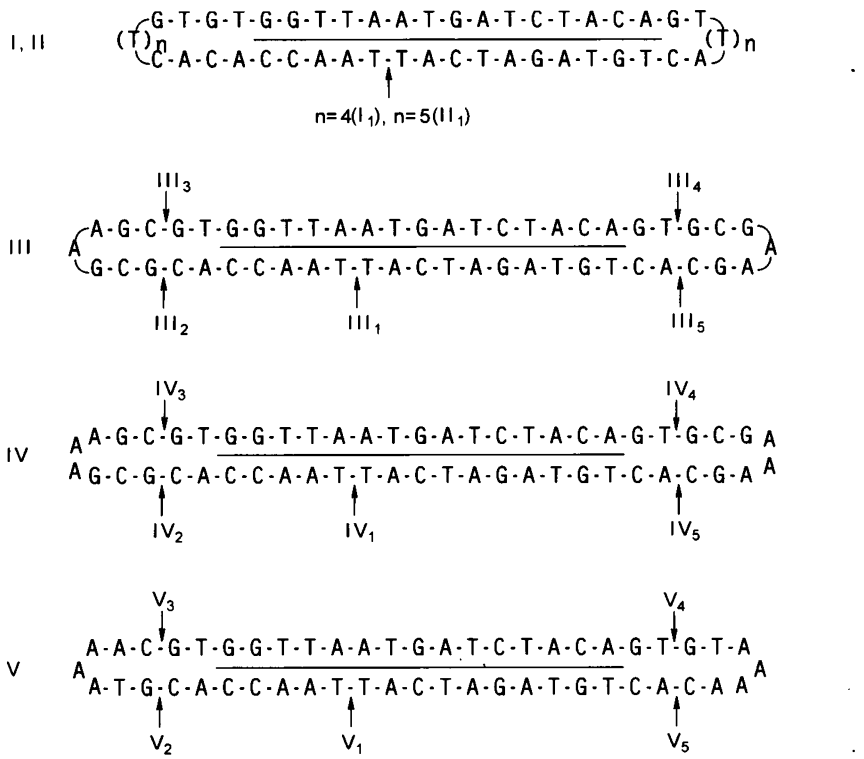

FIG. 1. Structures and sequences of nicked DNA dumbbells used in this study, as described in the text. The 15 bp site of HNF1 is underlined. Arrows indicate the positions of the nicks. Roman numerals with subscripts indicate the number of corresponding nicked DNA dumbbells. Each nicked DNA dumbbell contains only one nick. Roman numerals at left indicate the number of corresponding closed DNA dumbbells.

G-A (in the case of 5′-GCGA$_2$GC-3′; Hirao et al., 1994) base pairs. In addition, these highly stacked structures (Hirao et al., 1989) are strongly resistant to nucleases (Yoshizawa et al., 1994; Hirao et al., 1994).

The 5′-GTA$_4$C-3′ sequence was chosen as the closing fragment for nicked DNA dumbbells V_{1-5}. One G-C and one T-A base pair stabilize this fragment. An additional factor stabilizing the 5′-GTA$_4$C-3′ could be the stacking ability of the A_3-loop region (Powell et al., 1972; Olsthoom et al., 1980). As a control, we used nicked DNA dumbbells with T4 and T5 closing loops (I_1 and II_1), as they are the most stable structures with homonucleotide-containing loops (Blommers et al., 1989; Senior et al., 1988).

The position of the nick in the DNA dumbbell precursors and the nature of reacting nucleotide residues are important for the achievement of high nick-sealing efficiency. Placing the nick position between two T residues in the middle of the duplex stem (nicked DNA dumbbells with subscript 1) appear to be a rational strategy for at least two reasons. It provides for stable duplex formation in addition to the most productive contact at the ligation site (Dolinnaya et al., 1994). The other positions (nicked DNA dumbbells with subscripts 2–5) were chosen because of our interest in examining the efficiency of chemical ligation in close proximity to the loops. We have hypothesized that two base pairs of the minihairpins would be sufficient to bring the reactive ends sufficiently close to each other in the nick. Such nick positions could be useful for synthesis of short DNA dumbbells or when modified groups are located at the center of the duplex stem. The T3′-5′G, C3′-5′A, and C3′-5′G contacts are placed in the nick in these cases.

All polynucleotides that formed nicked DNA dumbbells contain 3′-end phosphate and 5′-end hydroxyl groups that were adjacent to one another in the ligation site (Shabarova, 1988; Dolinnaya et al., 1991).

Synthesis of covalently closed DNA dumbbells I–V

Covalently closed DNA dumbbells I–V are formed as a result of nick closing in the nicked precursors I_1, II_1, III_{1-5}, IV_{1-5}, and V_{1-5} (Fig. 1). EDC-induced and CNBr-induced chemical ligation was used for this purpose. These condensing agents were effective for synthesis of the phosphodiester bond. Reactions were performed as described in Materials and Methods.

The results of chemical ligations are summarized in Table 1. Figure 2 shows the electrophoretic analysis of the reaction mixtures formed as a result of CNBr-induced chemical ligation of the nicked DNA dumbbells. Ligation products were formed in all cases. Their cyclic structures were confirmed as described earlier (Clusel et al., 1993; Kuznetsova et al., 1996a). The ligation efficiency for DNA dumbells I–V was 10%–90% depended on the nature of the closing fragments, the nick position, and the nature of reacting nucleotide residues. Maximal yields were achieved when the nick was located in the middle of the molecule, between two T residues (70%–90%). This could be because of effective stabilization of the ligation site by the 9–14 bp duplex stems on both sides of the nick with one of

TABLE 1. EFFICIENCY OF CHEMICAL LIGATION IN NICKED DNA DUMBBELLS

No.	*Nicked DNA dumbbell number*	*Nucleotides facing the nick*	*Ligation efficiency, % (±5)*[a]	*Closed DNA dumbbell number*
1	I_1	TpT	90	I
2	II_1	TpT	70	II
3	III_1	TpT	85	III
4	III_2	CpG	80	
5	III_3	CpG	68	
6	III_4	TpG	73	
7	III_5	CpA	60	
8	IV_1	TpT	80	IV
9	IV_2	CpG	80	
10	IV_3	CpG	65	
11	IV_4	TpG	71	
12	IV_5	CpA	63	
13	$V1_1$	TpT	75	V
14	V_2	CpG	16	
15	V_3	CpG	10	
16	V_4	TpG	38	
17	V_5	CpA	12	

[a]Data presented are for CNBr-induced chemical ligation.

the most productive TpT contacts (Merenkova et al., 1992). Essentially similar results were obtained with nicked DNA dumbbells III_{2-5} and IV_{2-5}, which contained $GCGA_2GC$ and $GCGA_3GC$ minihairpin fragments. A ligation yield of 80% was achieved and was twofold higher than for linear DNA duplexes with the identical nucleotides facing the nick (Dolinnaya et al., 1994). It is worth noting that for nicked DNA dumbbells III_{2-5} and IV_{2-5}, the proximity of the reacting groups was provided from one hand only by two base pairs of the minihairpin sequences. The high ligation efficiency in these cases confirms the stabilizing effect of the $GCGA_2GC$ and $GCGA_3GC$ minihairpins on the double helix. We did not observe any significant differences in the coupling yields that depended on the nature of the nucleotides in the nicks, for example, CpG, TpG, and CpA. Essentially similar results were obtained earlier with linear DNA duplexes (Merenkova et al., 1992; Dolinnaya et al.,

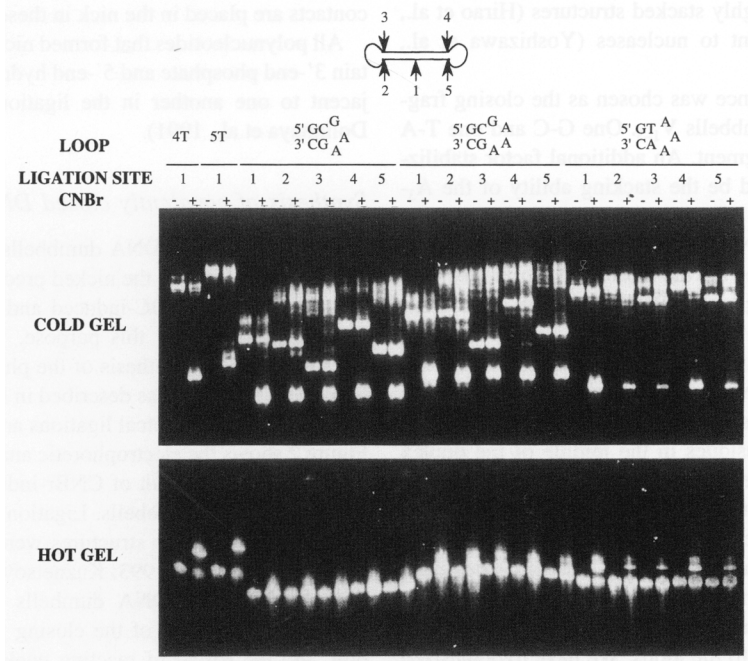

FIG. 2. Electrophoretic analysis of the reaction mixtures after CNBr-induced chemical ligation in nicked DNA dumbbells I_1, II_1, III_{1-5}–V_{1-5}. Detection was performed by EtBr staining of the gel. Schematic representation of the nicked DNA dumbbells is shown at the top. Arrows indicate the numbers of corresponding nicked DNA dumbbells. (−) or (+) indicates the absence or the presence of CNBr. Electrophoretic analysis was performed at room temperature (COLD GEL) and at 65°C (HOT GEL). Reactions were performed as described in Materials and Methods.

1994). However, along with the nicked DNA dumbbells mentioned previously, maximal yields (80%) were observed for DNA dumbbells III_2 and IV_2 with the CpG nick flanked from the guanosine side by minihairpins. For nicked DNA dumbbells III_3 and IV_3 containing the same contact but where guanosine residues were flanked by duplex stem, the ligation efficiency was lower. Our experimental data suggest that oscillation of the coupling yields may be due to a different orientation and proximity of the phosphate and hydroxyl groups in the ligation site. This may be caused by sequence-dependent modulation of the local structure of the DNA.

As compared with DNA dumbbells III_{2-5} and IV_{2-5}, the ligation efficiency of DNA dumbbells V_{2-5} containing the GTA_4C closing fragment was dramatically decreased (except V_4). In this case, the coupling yields were twofold less than for analogous linear DNA duplexes and more than fivefold less than for DNA dumbbells III_{2-5} and IV_{2-5}. The previous examples strongly suggest that the secondary structure of the closing sequence is the major factor that influences the efficiency of chemical ligation near the closing loops. The similarities between the coupling yields of linear DNA duplexes and dumbbells support the hypothesis that variations in chemical ligation efficiency arise from sequence-specific modulations in the helix geometry. It is worth noting that melting analysis of our nicked DNA dumbbells did not permit discrimination of a difference in the stability of DNA dumbbell V_{2-5} precursors before chemical ligation. The melting curves were similar for all nicked dumbbells mentioned (data not shown).

We discovered an interesting peculiarity in the electrophoretic mobility of the nicked and closed DNA dumbbells. As shown in Figure 2, the mobility of nicked DNA dumbbells compared with the closed ones strongly depends on the sequence of the closing fragments, the position of the nick, and the temperature of the gel. In a gel at ambient temperature, all closed DNA dumbbells migrated faster than their linear precursors. Maximal differences in mobility were achieved for DNA dumbbells I_1 and I, II_1, and II, and for V_{1-5} and V. For all DNA dumbbells containing the minihairpin sequences $GCGA_2GC$ and $GCGA_3GC$, this difference was smaller. Conceivably, this could be a result of the formation of more stable and more compact structures of these compounds. Moreover, in the two last cases, the difference in mobility of nicked and closed dumbbells depended on the nick position. It was greater for nick positions 1, 2, and 4, where the nicks are located in the middle of the molecule (position 1) or found symmetrically at the ends of pseudopalindromic double-stranded sequence-HNF1 binding site (positions 2 and 4). At the same time, for the analogous positions 3 and 5, the difference in mobility was about twofold less. Positions 2 and 4 differ from positions 3 and 5 only in the orientation of the minihairpins. Nicked DNA dumbbells III_2, IV_2, III_4, and IV_4 contain minihairpins at their 5′-ends, whereas III_3, IV_3, III_5, and IV_5 do so at the 3′-ends. We believe that this phenomenon requires additional study.

In a heated gel (65°C), the differences in mobility were greatly decreased. Moreover, in this case, closed DNA dumbbells migrated slower than the nicked ones (dumbbells I, II, and V) or at approximately the same level (dumbbells III and IV). In a heated gel, the position of the nick did not influence the mobility of the nicked DNA dumbbells (Fig. 2). Thus, knowledge of the dependence of electrophoretic mobility on gel condition is very important for successful separation of extended (50–54 member) nicked and closed DNA dumbbells.

Similar results were obtained for EDC-induced chemical ligation (data not shown). Such a correlation between the action of BrCN and EDC has been noted earlier for linear DNA duplexes (Merenkova et al., 1992; Dolinnaya et al., 1994). However, BrCN has some advantages over EDC, such as short reaction times and the absence of products with modified heterocyclic bases in the single-stranded region.

Several important conclusions can be drawn from these results. First, we found that chemical ligation in extended nicked DNA dumbbells can be efficient even if the nick is placed at a distance of two nucleotides from the end of the stem. The principal criteria for the design of precursors are to choose sequences that will preferentially adopt a stable hairpinlike structure as, for example, the $GCGA_2GC$ or $GCGA_3GC$ closing fragments. In this case, coupling yields were higher for CpG nucleotides facing the nick and were approximately the same as those observed for DNA dumbbells with the nick in the middle of the molecule and with the most productive TpT contact. Second, the use of CNBr decreased the reaction time and simplified product separation. Our approach also could be helpful in generation of small dumbbells and dumbbells containing modifications in the center of the stem. Knowledge of their electrophoretic mobility provides a solid foundation for the effective isolation of closed molecules. Thus, the rational design of DNA dumbbell precursor may lead to efficient, simple, and rapid large-scale synthesis of this class of molecule.

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