19. Short Optimally Capped Duplex DNA as Conformationally Restricted Analogue of B-DNA

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We describe the synthesis of short double-stranded DNA fragments (see **4** and **13**) which are capped on both ends by an optimally designed linker molecule. The new structures are stable with respect to hybrid dissociation and should have implications in physical studies involving double-stranded DNA as well as in the antisense area for the specific modulation of gene expressions.

1. Introduction. – Physical studies on DNA and DNA complexes with proteins/peptides or small molecules are mainly performed on oligonucleotide duplexes in order to decrease complexity. A drawback with oligonucleotides is that they often show a dynamic behavior with respect to hybridization, oligomer formation, and stem loop structures. One way to overcome these limitations is by cross-linking the two single-stranded DNA fragments. This may be achieved *via* internal disulfide cross-links, but this approach might disturb the instrinsic properties of the oligonucleotide double strand [1]. Another possible solution is cyclic DNA with a complementary region. However, this creates dumbbell-like structures with loop formation on both sides adjacent to the complementary region thus hampering optimal hybridization [2] [3] (*Fig. 1a*). A recently described stabilization of duplex DNA makes use of a hairpin structure which is cross-linked at the end by a disulfide bridge [4].

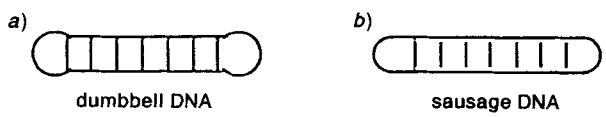


Fig. 1. *Schematic difference between dumbbell DNA and sausage DNA* (= σ-DNA)

To create optimally stabilized oligonucleotide duplexes, we sought for unnatural linker molecules which can bridge the 3′-OH and the 5′-OH function of both B-DNA duplex ends in such a way that the bases at the duplex ends are forced into an optimal position for hybridization. Thus, the resulting DNA duplex is capped on both ends (*Fig. 1b*) and represents a mimic of long double-stranded DNA. We call this type of DNA 'sausage DNA' (= σ-DNA) to distinguish it from 'dumbbell DNA'.

2. Results and Discussion. – 2.1. *Chemistry.* In search for optimal spacer molecules, we found that two propanediol units, interspaced by three phosphate groups, present an ideal linker as confirmed by molecular modelling (*Fig. 2*). Such a spacer unit can be introduced during solid-phase DNA synthesis by applying building block **1** (*Fig. 2*). Its synthesis was performed according to *Seela* and coworkers [5] and *Grollman* and coworkers [6] who also showed that introduction of this building block proceeds with high efficiency. Two units of **1** are used whenever the optimal linker has to be introduced.

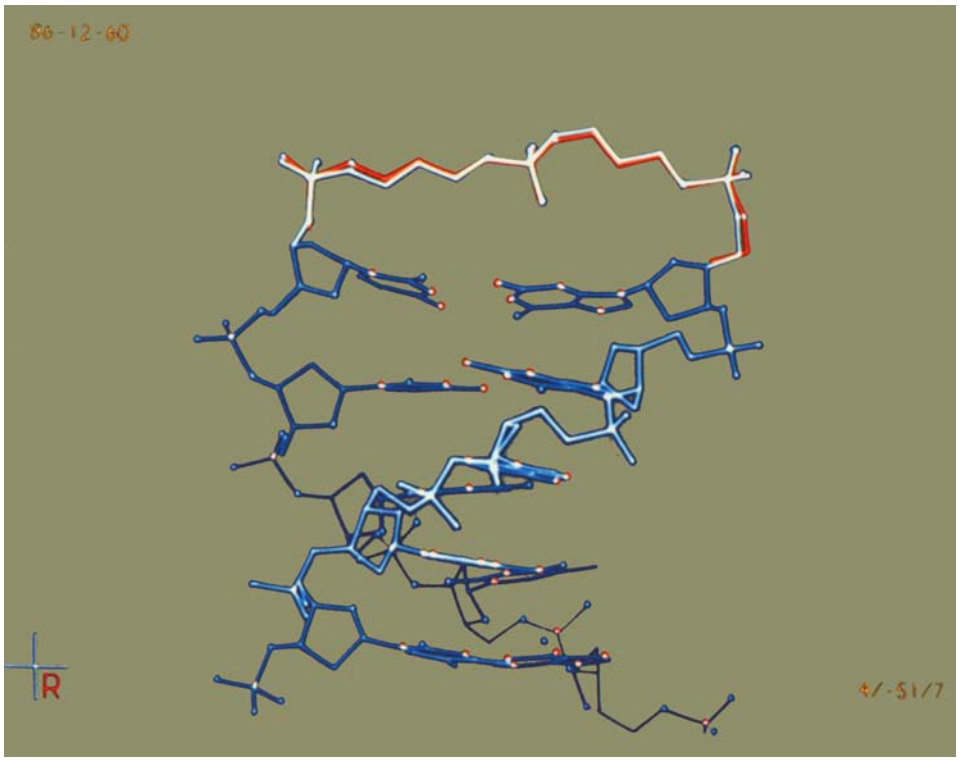
A problem is encountered in the ring closure leading to the cyclic unnatural DNA representing the sausage DNA. In principle, the cyclization can be performed with the protected DNA carrying a phosphodiester group at one end and an OH group at the other end of the fragment. Cyclization can then be mediated using solution phosphotriester methodology. For small cyclic DNA fragments, this approach was successfully used in solution chemistry by several groups [7–9]. With this approach, a major side reaction is oligomer formation [10]. Oligomerization can be prevented by attaching the exocyclic amino functions of the bases (either C, G, or A) to a suitable solid support and performing, after synthesis of the DNA fragment, the cyclization directly on the solid support [11].

Alternatively, this side reaction can be minimized, when the functional groups involved in the cyclization, namely the phosphate and the OH group, are brought into close proximity. Hence, the intramolecular reaction leading to the cyclic DNA is preferred over the intermolecular side reaction. This can be achieved with unprotected DNA due to the high specificity of the base pairing, as already demonstrated in a triple-helix arrangement for a chemical ligation of DNA fragments [12] and for the preparation of cyclic DNA by triple-helix stabilization of the reacting functionalities [13] [14].

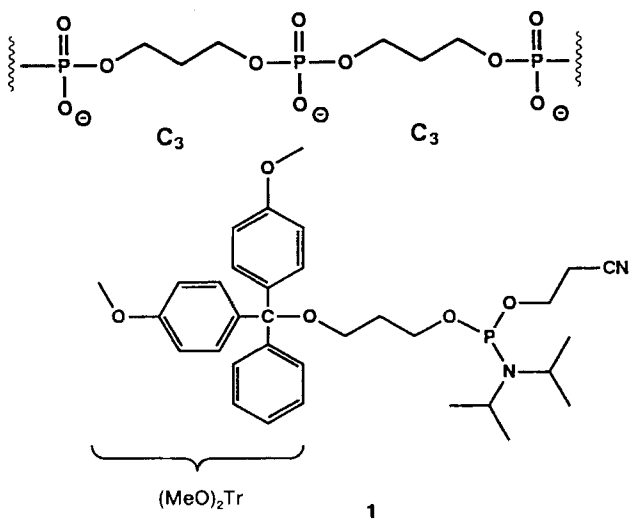
For the synthesis of 'sausage DNA', close proximity of the reacting functions can be achieved and stabilized as outlined in *Scheme 1*. The oligonucleotides **2** and **3** were synthesized in such a way that they can fold back from both the 3′- and the 5′-end placing the phosphate group and the OH group into an optimal position for cyclization. This is identical with the chemical approach for the preparation of dumbbell DNA which was reported recently by *Ashley* and *Kushlan* [3].

Fragment **2** was obtained by solid-phase phosphoramidite chemistry on controlled-pore glass (CPG) using a modified disulfide linker originally described by *Asseline* and *Thuong* [15]. For the introduction of the linker, standard phosphoramidites were replaced twice by **1** in the corresponding elongation cycles. After synthesis, the dimethoxytrityl ($(MeO)_2Tr$) group was removed and treatment with dithioerythritol (DTE)/NH_3 yielded the crude fragment **2** which was used after exchange of the NH_4^+ ions with K^+ without purification for the cyclization. Theoretically, all truncated sequences from the synthesis can cyclize, since they all carry the 3′-phosphate group. However, this might be prevented to a great extend due to the fixation upon back-hybridization resulting in larger distances between the phosphate and the OH function in the truncated sequences.

DNA fragment **3** was synthesized on deoxyguanosine-functionalized CPG. After assembly, the fragment was phosphorylated with bis(2-cyanoethoxy)(diisopropylamino)-phosphine as described earlier [16] [17]. Deprotection yielded crude **3**. Since capping is performed throughout the synthesis, only the desired fragment is phosphorylated and, therefore, the formation of smaller rings during cyclization is prevented.



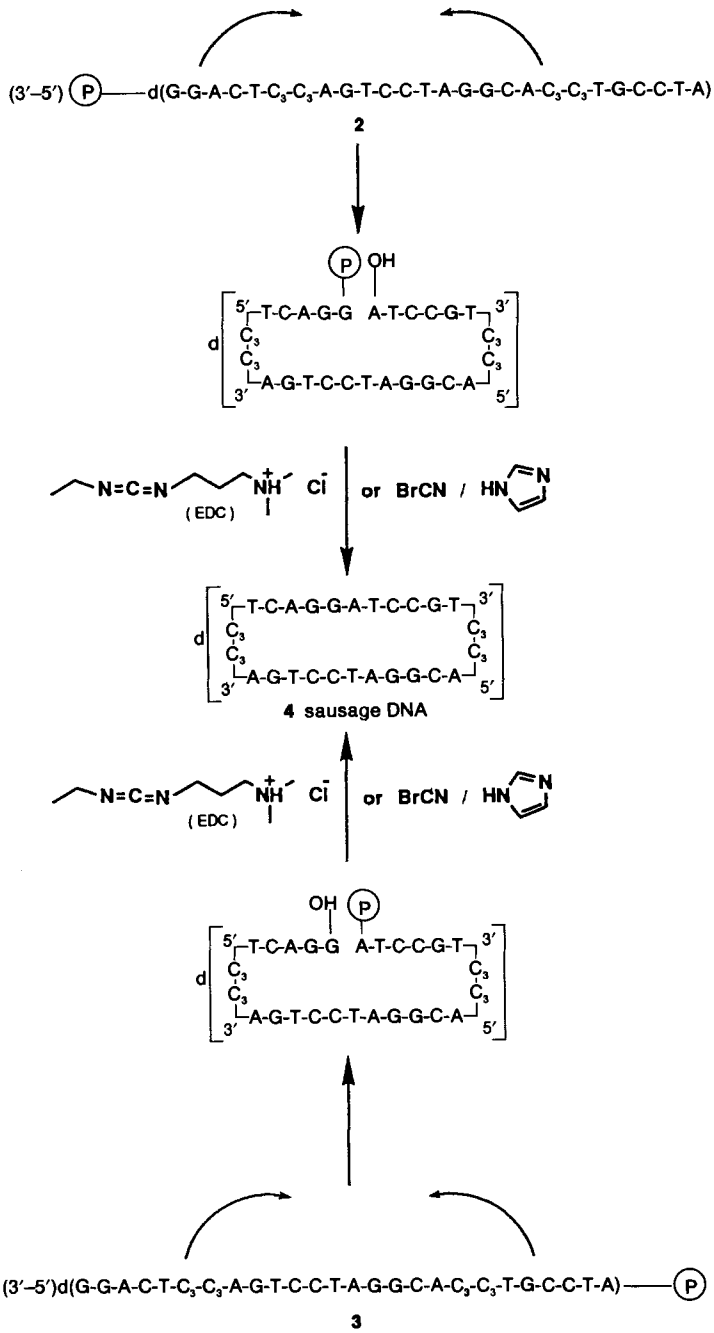
Spacer :



1

Fig. 2. *Molecular modelling of the end of a B-DNA duplex capped by the designed spacer molecule* (white, C·G end; orange, A·T end) *and building unit* **1** *for the insertion of the spacer molecule*

Scheme 1



P : phosphate group

C_3: linker unit

The ring closure to yield cyclic DNA can be performed by DNA ligase as previously demonstrated for the construction of dumbbell DNA [2] [18]. Enzymatic ligation, however, would limit the method to the preparation of relatively small amounts of the envisaged sausage DNA. Alternatively, the cyclization can be mediated by BrCN or *N*-[3-(dimethylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC). Both reagents were previously shown to be suited for the chemical ligation process of a phosphomonoester with an OH function [3] [19]; furthermore, they are cheap, and the scale of the ligation is not limiting. However, it still had to be established that these condensing reagents are also suitable for the effective conversion of the linear fragments to the sausage DNA.

Following the strategy outlined above, we observed, by PAGE (polyacrylamide-gel electrophoresis (20%), denaturing conditions), no significant difference in cyclization starting either from 3'-phosphate **2** or 5'-phosphate **3** when using BrCN for the activations (*Fig. 3*). When EDC was applied to this step, a higher efficiency was obtained for the

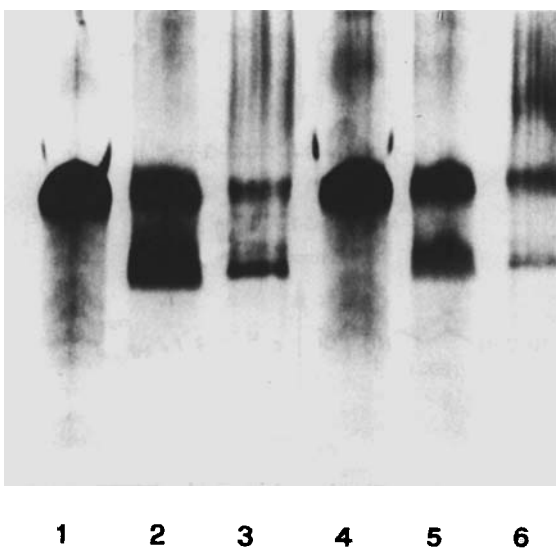


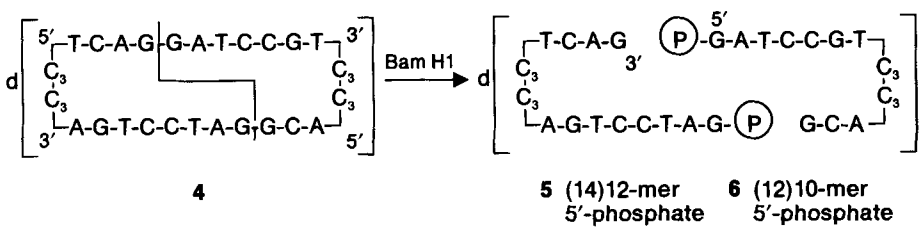
Fig. 3. *PAGE* (20%; denaturing conditions) *of cyclization mixtures*: Lane 1: *crude* **2** *for cyclization*; Lane 2: *cyclization of* **2** *by EDC*; Lane 3: *cylization of* **2** *by BrCN*; Lane 4: *crude* **3** *for cyclization*; Lane 5: *cyclization of* **3** *by EDC*; Lane 6: *cyclization of* **3** *by BrCN*

cyclization of **2** than for **3**. In later cyclizations, we realized that further addition of condensing reagent leads to quantitative formation of **4**. This complete conversion should simplify large-scale preparations of sausage DNA.

2.2. *Characterization.* The degradative characterization of sausage DNA is a problem due to its resistance to exonucleases. Fragment **2** was degraded by spleen phosphodiesterase, whereas **4** was completely stable towards this exonuclease (data not shown). In gel-electrophoresis analysis, a significant increase in mobility was observed for σ-DNA when compared to the unligated precursors **2** and **3** (*Fig. 3*).

To obtain a more accurate characterization of σ-DNA, the recognition sequence for the endonuclease Bam H1 was inserted into **4**. Upon cleavage with this restriction enzyme, two fragments of different size (**5** and **6**) were expected. These fragments **5** and **6** were synthesized independently and then shown in gel electrophoresis to have the same mobility as the cleavage products of **4** with Bam H1 (*Scheme 2*; *Fig. 4*). The weak bands

Scheme 2



4

5 (14)12-mer 5′-phosphate **6** (12)10-mer 5′-phosphate

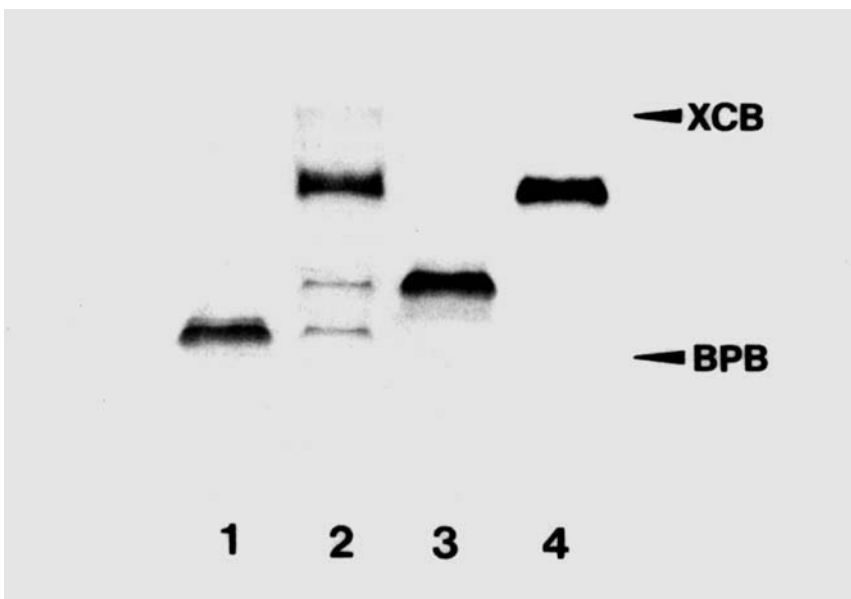


Fig. 4. *PAGE* (20% denaturing conditions) *of Bam H1 digest of* **4**; Lane 1: **6**; Lane 2: *digest of* **4** *with Bam H1*; Lane 3: **5**; Lane 4: **4** (XCB = Xylene Cyanol FF, BPB = Bromophenol Blue)

with lower mobility than **4** correspond to the fragments obtained by singly cleavage with the enzyme. As a control, the unligated products **2** and **3** were also digested with Bam H1. Surprisingly, the enzyme accepted the nick in **2** and **3** and produced fragments of different size as compared to the cleavage of **4**. Thus, an additional indication for the right structure of the sausage-type DNA was obtained.

2.3. *Melting Behavior.* The melting behavior of the sausage DNA **4** in comparison with the duplexes **7**–**9** was investigated (*Fig. 5*). This analysis would show the stabilizing

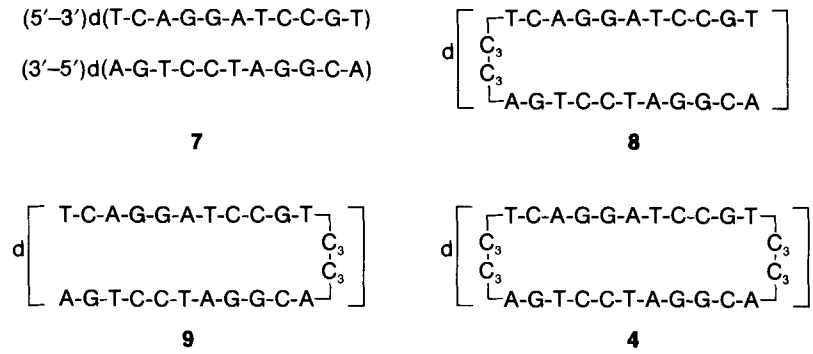


Fig. 5. *Duplexes of which the melting temperatures were determined*

effect of the linker units on the hybridization of the duplex DNA. The uncapped duplex **7** shows a melting temperature of *ca.* 45°. Introduction of a linker at one end of the duplex as in **8** and **9** leads to an increase in melting temperature of *ca.* 10°. If both ends of the duplex are capped as in σ-DNA **4**, no melting can be observed up to 85°, thus confirming the expected stabilizing effect of the optimal linker (*Fig. 6*). As deduced from molecular modelling, the phosphate group in the linker can form a H-bridge with the G of a GC pair at the end of the double helix.

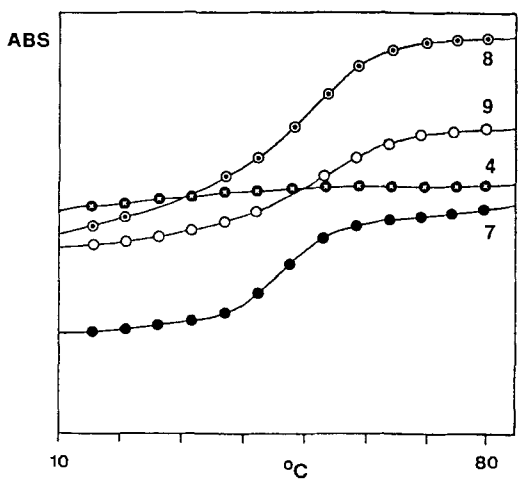
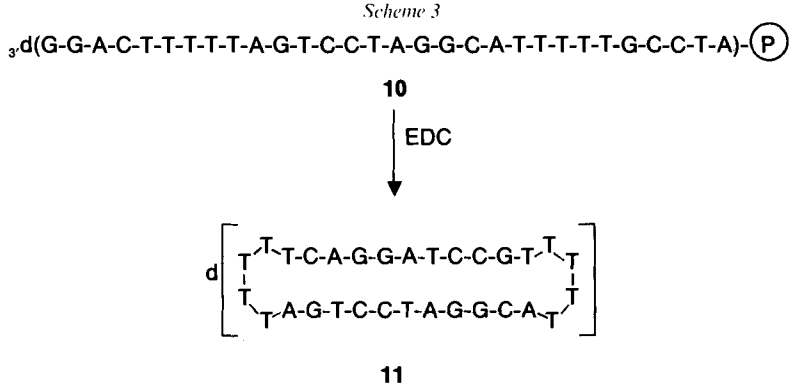


Fig. 6. *Melting curves of the oligomers* **4** *and* **7**–**9** (ABS = UV absorption at 260 nm)

To further elaborate the stabilizing effect of optimally designed linker molecules, we synthesized the corresponding dumbbell DNA **11** from **10** according to *Scheme 3* and compared its melting profile with that of **4**. The result (*Fig. 7*) shows the enhanced stability of **4**.

Scheme 3



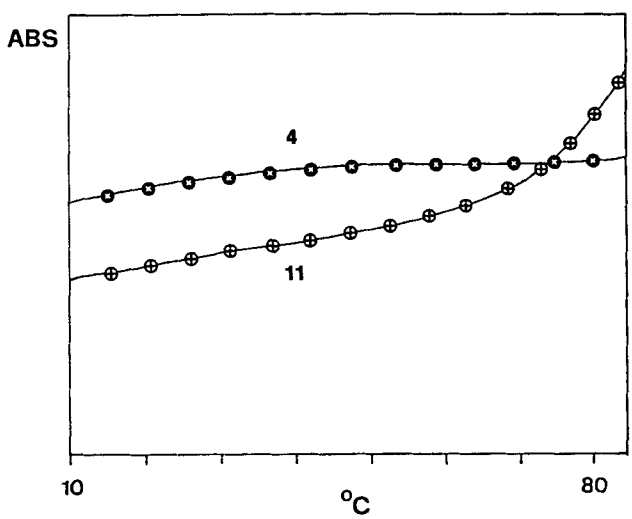
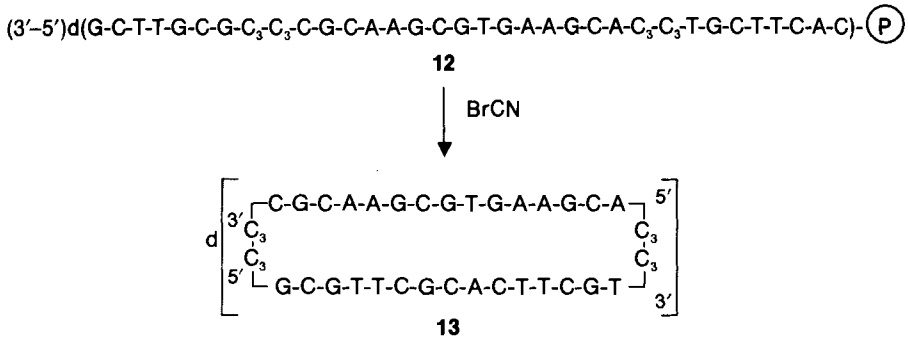


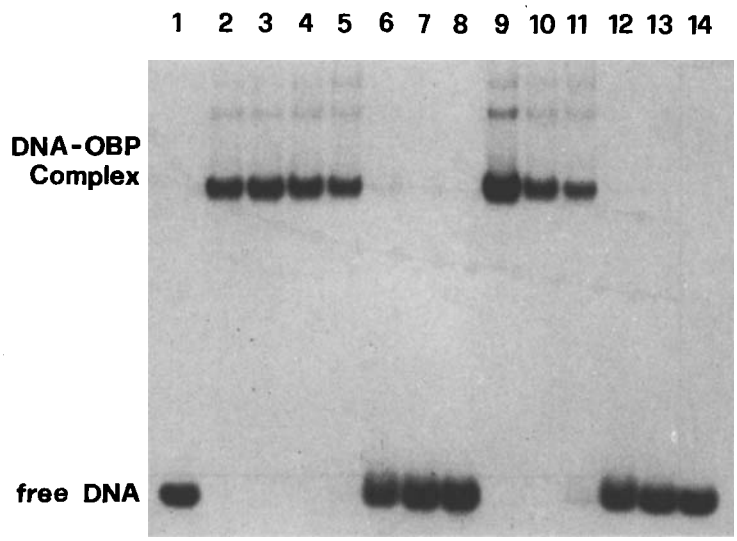
Fig. 7. *Melting curves of* **4** *in comparison with* **11** (ABS = UV absorption at 260 nm)

2.4. *Protein Binding.* One of the many possible applications of sausage DNA will be studies of protein-DNA interactions. To test the binding of proteins to σ-DNA, we synthesized the σ-DNA **13** from **12** by the strategy outlined above (*Scheme 4*). σ-DNA **13** represents one of the two binding sites (box I) of the origin-binding protein (OBP) of *Herpes simplex* virus type 1 (HSV-1) at the origin of replication ori$_s$ [20] [21]. The OBP, a product of the UL9 gene, is a protein 851 amino acids in length, of which only 269 or less amino acids near the C-terminus are required for sequence-specific recognition of the origin of replication [22].

In our experiments, we used a polypeptide containing the C-terminal 317 amino acids of OBP. It was expressed in *E. coli* and partially purified on a FPLC anion-exchange *Mono-Q™-HR-5/5* column. Using competition experiments based on the mobility-shift assay [23], the interactions between OBP and either a duplex oligonucleotide **14**·**15** (HSV ori17) or the sausage DNA **13** were determined (*Fig. 8*). Both DNA fragments contain the

Scheme 4





HSV ori17: (5′–3′)d(A-G-C-G-T-T-C-G-C-A-C-T-T-C-G-T-C) **14**

(3′–5′)d(T-C-G-C-A-A-G-C-G-T-G-A-A-G-C-A-G) **15**

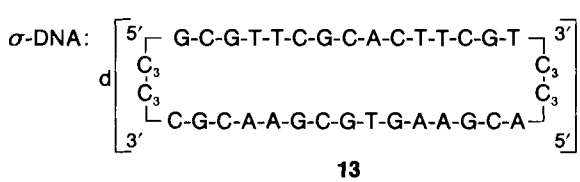


Fig. 8. *PAGE* (10%; native conditions) *study of binding of the OBP to the duplex*, **14**·**15** (*Lanes 3–8*) *and to the sausage DNA* **13** (*Lanes 9–14*). Concentrations of unlabelled DNA's are 10 fmol (*Lanes 3* and *9*, resp.), 50 fmol (*Lanes 4* and *10*, resp.), 100 fmol (*Lanes 5* and *11*, resp.), 1 pmol (*Lanes 6* and *12*, resp.), 5 pmol (*Lanes 7* and *13*, resp.), and 10 pmol (*Lanes 8* and *14*, resp.). Controls are free HSV ori17 (*Lane 1*) and OBP-HSV ori17 complex (*Lane 2*).

high-affinity binding site box I of OBP. OBP from *Mono-Q* column fractions were incubated for 20 min at room temperature with ^{32}P end-labeled [32p] HSV-ori17 oligonucleotide and increasing concentrations of either unlabelled HSV ori17 or σ-DNA **13**. The samples were loaded on a 10% polyacrylamide gel, electrophoresed under native conditions, dried, and autoradiographed. *Fig. 8* shows the result of such an experiment. Retarded bands representing DNA-protein complexes were detected. The displacement of OBP from its DNA recognition site can be observed at equal concentrations (1 pmol) for both competitor DNA's, the duplex oligonucleotide HSV ori17 and the σ-DNA **13**. This indicates a similar binding affinity of the peptide to the terminally 'open' and 'closed' DNA.

3. Conclusion and Outlook. – To stabilize short DNA duplexes, we developed optimal linkers which force the bases at the duplex ends to hybridize without disturbing the intrinsic properties of the duplex. The linkers lead to highly stabilized structures with respect to hybrid dissociation. This stabilized duplex DNA to which we refer as sausage DNA ($= \sigma$-DNA) can be efficiently prepared by chemical means. The linker can be incorporated in standard solid-phase DNA synthesis by the phosphoramidite approach. Chemical ligation can be performed with high efficiency starting from the crude linear DNA fragments containing the linker units and which are either phosphorylated at the 5′- or the 3′-end. A further stabilization of the σ-DNA can be achieved if the linker unit contains entities which are able to form H-bridges with the bases at the duplex end, and a further stabilization effect can possibly be achieved with linkers containing aromatic units due to base-stacking interactions with the bases at the end of a sausage DNA.

To identify the correct structure of the prepared sausage DNA **4**, we inserted into **4** the recognition site for Bam H1. Incubation of **4** with this enzyme yielded the expected fragments which were synthesized independently for comparison. Melting curves confirmed the high stabilization afforded by the optimal linker. This becomes also obvious when comparing the melting behavior of the dumbbell DNA **11** with the corresponding sausage DNA **4**. The specific binding of a protein molecule to sausage-type DNA was demonstrated for the binding of the origin-binding protein of *Herpes simplex* virus type 1 to DNA **13** containing the high-affinity binding site of the protein. The concept of using sausage-type DNA instead of long double-stranded DNA should make it easier to study by physical methods the interactions between double-stranded DNA and other molecules (low molecular weight binders, peptides, proteins). Furthermore, the high stability of σ-DNA allows for great flexibility with respect to the conditions that can be used in these investigations. The molecules are also resistant towards exonucleases.

Sausage DNA (σ-DNA) should represent an ideal tool for both qualitative and quantitative studies of DNA-ligand complex formation, to study triple-helix formation, to discriminate between exo- and endonuclease activities, and as transcription modulators.

During the preparation of this manuscript, we became aware that this linker approach was also applied by others for RNA miniduplexes of the HIV-1 TAR element [24].

We would like to thank Prof. *Klaus Müller* and Dr. *Daniel Bur* for modelling and Dr. *Eric Kitas* for critical reading of the manuscript.

Experimental Part

1. *General.* All solvents were of highest purity available. The 1,4-dithioerythritol (= *erythro*-1,4-dimercaptobutane-2,3-diol; DTE), BrCN, *N*-[3-(dimethylamino)propyl]-*N'*-ethyl carbodiimide hydrochloride (EDC), 4,4'-dimethoxytriphenylchloromethane ($(MeO)_2TrCl$), and 2,2'-dithiodiethanol were all from *Fluka*. Restriction enzyme Bam H1, 'one phor all' buffer and *NAP10-Sephadex* columns were all from *Pharmacia*. [γ-^{32}P]ATP (3000 Ci/mmol) was from *Amersham*. Short column chromatography (CC) [25]: silica gel *60* (0.063–0.040 mm; *Merck*). Fragments **14** and **15** were from *Microsynth*. UV-Absorption melting curves: *Perkin-Elmer-λ2* photometer connected with a *PTP-6 Peltier* temp. programmer.

2. *Synthesis of DNA Fragments.* Phosphinylating reagent bis(2-cyanoethoxy)(diisopropylamino)phosphine was prepared according to [17]. DNA fragments d(T-C-A-G-G-A-T-C-C-G-T) and d(A-C-G-G-A-T-C-C-T-G-A) were prepared on controlled-pore glass (CPG; *Sigma*) as solid support [26] applying a 10-fold excess of (2-cyanoethyl) phosphoramidites [27] and a 130-fold excess of 1*H*-tetrazole using our standard technologies [28]. Solid support for the synthesis of fragment **2** was essentially prepared as mentioned in [15] but using sarcosine-modified CPG [29] for the attachment of the disulfide linker. Functionalization as determined by $(MeO)_2Tr$ cleavage and UV measurement: 38 μmol/g of support.

Building block **1** was prepared by modifying a published procedure [4]: Propane-1,3-diol was first reacted with $(MeO)_2TrCl$ to yield 3-[(4,4'-dimethoxytrityl)oxy]propan-1-ol. The compound could only be obtained in crystalline form after CC. Yield 51%, m.p. 71–72° ([5]: 69–70°). Subsequent phosphinylation was performed with (2-cyanoethoxy)bis(diisopropylamino)phosphine with diisopropylammonium tetrazolide activation [17]. Yield after CC 89%. ^{31}P-NMR: 148.5 ppm ($CDCl_3$; rel. to H_3PO_4).

Fragment **2**: Preparation was performed starting with 3.2 μmol of the modified support mentioned above. Whenever the linker unit had to be introduced, two successive couplings with building block **1** were carried out applying standard cycles. After complete assembly, the $(MeO)_2Tr$ group was removed. Further deprotection and release as 3'-phosphate was by treatment with 1 ml of 0.2M DTE in conc. NH_3 soln. for 2 h at 70°. Part of the material was analyzed by gel electrophoresis to assess the performance of the solid-phase synthesis. Crude **2** was obtained after evaporation of the NH_3 soln. on a *speed-vac* concentrator. After precipitation from 400 μl of 80% AcOH and 1 ml of Et_2O, it was evaporated several times after addition of KCl. Excess of KCl was removed by *Sephadex* filtration and the fragment used as such for the cyclization.

Fragment **3**: Preparation was performed starting from CPG functionalized with dG (2.7 μmol). Elongations and incorporation of the linker was as described for **2**. After assembly of the chain, the $(MeO)_2Tr$ group was cleaved off and phosphorylation performed with bis(2-cyanoethoxy)(diisopropylamino)phosphine as described in [17]. Deprotection was performed with 1 ml of conc. NH_3 soln. at 70° for 2 h. Workup and transfer into the K^+ salt was performed as mentioned above. Gel electrophoresis showed an efficient synthesis of the oligomer and, therefore, the crude fragment was used for the cyclization.

Fragments **5**, **6**, *and* **12** which also contain linker units and which are phosphorylated at the 5'-end were synthesized accordingly.

Fragment **10** was prepared in a standard fashion followed by phosphorylation on the solid support as mentioned above.

Fragments **8** *and* **9** were synthesized similarly, except that the phosphorylation after assembly of the chain was omitted.

3. *Cyclizations. Conversion of* **12** *to* **13** *with BrCN (General Procedure)*: A stock soln. of 10 μl of crude **12** (corresponding to *ca.* 15 nmol) in the K^+ form was mixed with 4 μl of 500 mM $NiCl_2$, 4 μl of 1M 1*H*-imidazole/HCl pH 7.0, and 4 μl of 250 mM BrCN. After 24 h at r.t., conversion was *ca.* 50% as determined by gel electrophoresis. Another 4 μl of BrCN soln. was added and allowed to react for a further 24 h to almost quantitatively yield **13**. The volume of the mixture was made up to 100 μl with H_2O and the DNA precipitated after addition of 200 μl of dioxane and 600 μl of THF. Purification was performed by denaturing 20% polyacrylamide-gel electrophoresis followed by H_2O extraction or electroelution.

Conversion of **2** *to* **4** *with EDC* [3] [19] *(General Procedure)*: The crude DNA fragment **2** in the K^+ form was cyclized in 20 μl of reaction mixture for 24 h at r.t. (50 mM morpholine-4-ethanesulfonic acid (MES; pH 6.0), 20 mM $MgCl_2$, 400 mM EDC). Further workup and purification was performed as mentioned above.

Cyclization of **10** to **11** was performed accordingly.

4. *Enzymatic Digestion.* To the sausage DNA **4** (0.2 OD_{260}) in 20 μl of H_2O were added 3 μl of 10× 'one phor all' buffer and 10 μl (200 U) of Bam H1. Incubation proceeded at 37° for 20 h. After denaturing for 2 min at 95°, the mixture was analyzed by anal. denaturing 20% polyacrylamide gel electrophoresis (PAGE) in which the DNA was

made visible by 'stains-all' (*Fig. 4*). The same incubation was also performed with the open-chain oligomers **2** and **3** which resulted in cleavage fragments of different size as compared to the cleavage of **4**. In parallel, the activity of the enzyme was confirmed by cleavage of a duplex DNA containing a Bam H1 site.

5. *Melting Curves. Ca.* 0.7 OD_{260} of DNA fragments **8**, **9**, and **4** or a mixture **7** of 0.6 OD_{260} of d(T-C-A-G-G-A-T-C-C-G-T) and d(A-C-G-G-A-T-C-C-T-G-A) were taken up in an UV cell in 1 ml of 5 mM phosphate buffer pH 7.0, 100 mM NaCl, and 0.1 mM EDTA and covered with pentadecane. The UV absorbance was measured at 260 nm from 10 to 90° with a temp. increase of 0.5° per min (see *Fig. 6*).

Similarly, the dumbbell DNA **11** was compared with the sausage DNA **4** (see *Fig. 7*).

6. *Protein Binding.* Using the mobility-shift assay [23], we studied the binding efficiency of the origin-binding protein (OBP) of *Herpes simplex* virus type 1 to the double-stranded oligonucleotide (17 b.p.) HSV ori17, representing the high-affinity binding site of OBP. Binding was compared to the sausage DNA **13**, and analogue of the same site (*Fig. 8*). OBP and labelled [^{32}P] HSV ori17 (10 fmol; 1600 cpm/ml) were incubated with increasing concentrations of either unlabelled HSV ori17 (*Lanes 3–8*) or unlabelled **13** (*Lanes 9–14*) for 30 min at r.t., electrophoresed on a native 10% polyacrylamide gel, and autoradiographed.

REFERENCES

[1] A. E. Ferentz, G. L. Verdine, *J. Am. Chem. Soc.* **1991,** *113,* 4000.
[2] B. C. F. Chu, L. Orgel, *Nucleic Acids Res.* **1991,** *19,* 6958.
[3] G. W. Ashley, D. M. Kushlan, *Biochemistry* **1991,** *30,* 2927.
[4] G. D. Glick, *J. Org. Chem.* **1991,** *56,* 6746.
[5] F. Seela, K. Kaiser, *Nucleic Acids Res.* **1987,** *15,* 3113.
[6] M. Takeshita, C.-N. Chang, F. Johnson, S. Will, A. P. Grollman; *J. Biol. Chem.* **1987,** *21,* 10171.
[7] E. de Vroom, H. J. G. Broxterman, L. A. J. M. Sliedregt, G. A. van der Marel, J. H. van Boom, *Nucleic Acids Res.* **1988,** *16,* 4607.
[8] M. L. Capobianco, A. Carcuro, L. Tondelli, A. Garbesi, G. M. Bonora, *Nucleic Acids Res.* **1990,** *18,* 2661.
[9] M. V. Rao, C. B. Reese, *Nucleic Acids Res.* **1989,** *17,* 8221.
[10] A. F. Markham, E. Nakagawa, E. Ohtsuka, M. Ikehara, *Chem. Pharm. Bull.* **1979,** *27,* 2988.
[11] S. Barbato, L. De Napoli, L. Mayol, G. Piccialli, C. Santacroce, *Tetrahedron* **1989,** 4523.
[12] K. J. Luebke, P. B. Dervan, *J. Am. Chem. Soc.* **1989,** *111,* 8733.
[13] E. T. Kool, *J. Am. Chem. Soc.* **1991,** *113,* 6265.
[14] G. Prakash, E. T. Kool, *J. Chem. Soc., Chem. Commun.* **1991,** 1161.
[15] U. Asseline, N. T. Thuong, *Tetrahedron Lett.* **1989,** *30,* 2521.
[16] E. Uhlmann, J. Engels, *Tetrahedron Lett.* **1986,** *27,* 1023.
[17] W. Bannwarth, A. Trzeciak, *Helv. Chim. Acta* **1987,** *70,* 175.
[18] M. Wolters, B. Wittig, *Nucleic Acids Res.* **1989,** *17,* 5163.
[19] Z. A. Shabarova, I. N. Merenkova, T. S. Oretskaya, N. I. Sokolova, E. A. Skripkin, E. V. Alexeyeva, A. G. Balakin, A. A. Bogdanov, *Nucleic Acids Res.* **1991,** *19,* 4247.
[20] P. Elias, I. R. Lehman, *Biochemistry* **1988,** *85,* 2959.
[21] P. D. Olivo, N. J. Nelson, M. D. Challberg, *Proc. Natl. Acad. Sci. U.S.A.* **1988,** *85,* 5414.
[22] S. Deb, S. P. Deb, *J. Virol.* **1991,** *65,* 2829.
[23] A. Dorn, M. Affolter, M. Muller, W. J. Gehrig, W. Leupin, *EMBO J.* **1992,** *11,* 279.
[24] M.-X. Ma, L. McCallum, S. C. Climie, R. Kuperman, W. C. Lin, M. Sumner-Smith, R. W. Barnett, *Nucleic Acids Res.* **1993,** *21,* 2585.
[25] B. J. Hunt, B. Rigby, *Chem. Ind. (London)* **1967,** 1868.
[26] S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder, G. R. Galluppi, *J. Am. Chem. Soc.* **1983,** *105,* 661.
[27] N. D. Sinha, J. Biernat, J. McManus, H. Koester, *Nucleic Acids Res.* **1984,** *12,* 4539.
[28] W. Bannwarth, *Chimia* **1987,** *41,* 302.
[29] T. Brown, C. E. Pritchard, G. Turner, S. A. Salisbury, *J. Chem. Soc., Chem. Commun.* **1989,** 891.