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A FUNCTIONAL APPROACH TO THE MAPPING OF STRUCTURAL POLYMORPHISMS IN SUPERHELICAL DNA

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We have constructed derivatives of the pGEM™-1 plasmid (pCC101, pCC102) which contain the sequences $d(C-G)_7$ or $d(T-G)_7$ in the polylinker region (e.g. the selfcomplementary oligonucleotide AATTCGCGCGCGCGCG was recombined with Eco RI restricted pGEM™-1). At physiological ionic strength and natural superhelical density the recombined "Py-Pu"-sequences flip into Z-conformation. This was reconsidered by immunoprecipitation of a complex of plasmid and monoclonal anti-Z-DNA-antibody (Z22, kindly provided by A. Rich, MIT) [1] and by carbethoxylation with diethylpyrocarbonate (DEPC) [2]. The polylinker region is flanked by the strong promoters for SP6 and T7 RNA polymerase. Additionally, we synthesized two $d(N)_{20}$ oligonucleotide-primers which are complementary to the "lower" and "upper" strand sequences, resp., flanking the polylinker. Hybridization of the superhelical plasmids with one of these primers creates a stable hybrid (a so called D-loop) which in turn serves as a primed template pointing towards the polylinker. The Klenow-fragment of DNA-Polymerase I as well as Reverse Transcriptase (AMV) both accept the D-loop primer and "replicate" the template strand.

The reaction was performed as follows: 1 μg of plasmid DNA (purified on CsCl density gradient) was incubated at 75 °C for 1 h in a total volume of 10 μl including 1.3 μl 10-fold concentrated "High salt Klenow buffer" (50 mM $MgCl_2$, 1.5 M NaCl, 100 mM Tris-HCl pH 8.5) and 170 kBq of 5′-[^{32}P]-endlabelled primer. After a short spin the primer-D-loop-hybrid was immediately cooled on ice, and 3 μl of a mixture containing 1 unit Klenow-fragment (Pharmacia) and all four deoxynucleotide-triphosphates (final concentration of each dNTP 0.4 mM!) were added. Following incubation at 37 °C for 30 min, the reaction was stopped by addition of 6 μl formamide dye mix (95% (v/v) deionized formamide, 20 mM EDTA, 0.1% (w/v) bromphenolblue, 0.1% (w/v) xylene cyanol), and the DNA was denatured at 95 °C for 3 min. 2–5 μl of the sample were electrophoresed on a 10% sequencing gel in Tris-borate buffer at 75 W. The same conditions were used for the reaction with Reverse Transcriptase (AMV, Life Science). 5 units of the enzyme were provided to compensate for the not optimal buffer conditions. Topoisomeres were generated by the standard protocol of Singleton and Wells [3] by the use of Ethidiumbromide and Topoisomerase I (BRL-Gibco).

The Z-conformation of the DNA template at the sequences $d(C-G)_7$ and $d(T-G)_7$ causes a specific pattern of synthesis stops (or pauses) which display bands in an autoradiograph with both enzymes tested. Surprisingly, the Klenow enzyme and the Reverse Transcriptase detect, besides Z-stretches, other local structural polymorphisms. The rate of the Klenow-polymerase was not significantly affected by changes by variation of salt and pH in the range of 2–20 mM $MgCl_2$, 0–200 mM NaCl and pH 7.5–8.5.

The DNA-dependent polymerization can also be performed after a Z-DNA specific antibody has bound to the Z forming sequences. A polyclonal, high affinity anti-Z-DNA-antibody preparation [4] modifies the stop pattern at distinct sites and its influence exhibits a dependence on superhelical density.

The presence of the SP6 and T7 RNA polymerase allows for transcription of the superhelical

template through the region of interest from both directions. The phage RNA polymerases do not stop at the Z-conformation neither in the presence nor in the absense of anti-Z-DNA-antibody. This is, at least in part, a contratiction compared to the results of Peck and Wang [5] who described a transcriptional block for RNA Polymerase of E. coli; however, in their case only $(C\text{-}G)_{16}$ stopped transcription of this enzyme.

The use of D-loop primers allows for mapping of local structural DNA polymorphisms and of specific protein binding in every superhelical DNA of interest. This method offers a functional (and probably more meaningful) alternative to chemical modification studies. It may lead to the elucidation of a relation between base sequence and local structural polymorphisms, as our preliminary results with native SV40 DNA indicate.

At present we do not understand the molecular origin of the observed stop patterns. On the basis of control experiments we can, however, exclude that "simple" effects like starvation for a dNTP or secondary structures (well known from Sanger sequencing artefacts) are involved. Since one necessary condition for D-loop formation is a negatively superhelical DNA we believe that superhelicity evokes the signal.

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