Nucleic Acids Research, Vol. 20, No. 21 5853–5854

The use of purine-rich oligonucleotides in triplex-mediated DNA isolation and generation of unidirectional deletions

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Received September 23, 1992; Accepted October 7, 1992

Recent reports (1, 2) describe a direct DNA isolation method that is based on the formation of intermolecular triple-helix (triplex) between double-stranded target DNA and a single-stranded oligonucleotide probe, thus termed 'triplex affinity capture'. This method utilizes biotinylated homopyrimidine probes that are incubated with the target DNA under acidic pH and high salt conditions to form triplex complexes, which are then separated from bulk DNA by use of streptavidin-coated magnetic beads. Extension of the technique, however, is hampered by the required use of low pH and high salt buffers which hinder combined usage of enzymes or other biological interactions. Here we demonstrate that purine-rich oligonucleotide probes composed of G and T bases form stable and specific triplexes with target DNA under physiological conditions, and can be used with enzymatic manipulations as well as potentially broader applications which require the rapid separation of DNA while retaining natural DNA-protein interactions and other biological functions.

As previously described, homopurine tracts on a double-stranded DNA can form Hoogsteen hydrogen bonds with homopyrimidine (3) or purine-rich (4, 5) oligonucleotides in parallel or antiparallel orientations, respectively. We constructed a plasmid vector (Figure 1) that carries a homopurine target for triplex formation juxtaposed to a multiple cloning site. This plasmid was cleaved into two fragments by restriction enzymes, and was directly incubated with a 5′-biotinylated purine-rich GT probe (see Figure legend for detailed configuration). By affinity capture of the probe with streptavidin-coated magnetic beads, we were able to specifically isolate, as triplex complex, the fragment that contained the target sequence but not the control fragment containing an unrelated homopurine tract (lanes 4, 5). Formation of the triplex between purine-rich GT probe and target requires the presence of multivalent cations such as Mg^{2+} at physiological levels, but appear to be independent of pH (5) or salt conditions (data not shown), and therefore, compatible with most enzyme reaction conditions. In contrast, the homopyrimidine-probe method required exchange of buffer to acidic pH after enzyme treatments, and also the presence of SDS to exclude nonspecific interaction (compare lanes 2 and 3) if high salt buffers (such as 2M NaCl) are not used. Neither SDS nor high salt is essential for the specificity of the triplex formed by purine-rich GT probes.

The broad compatibility of the purine-rich-GT-probe method also allows the combined usage of the two probe types in a multiplex isolation system. Both the purine-rich and the homopyrimidine probes are incubated with double-stranded DNAs in acidic buffer so that they each form triplex complexes with their respective targets. After these complexes are collected by biotin-streptavidin-mediated magnetic separation, double-stranded target can be released from the homopyrimidine probe

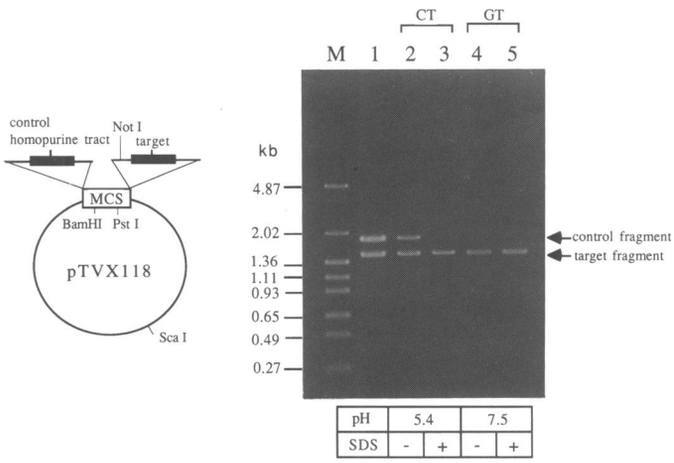

Figure 1. Design of the 'triplex' vector and agarose gel electrophoresis demonstrating the isolation of target fragment by triplex affinity capture. Fragments containing either a 26 bp homopurine target sequence (5′$GAG_3AG_2AG_4AGAG_2(AG_3)_23$′) or an unrelated 26 bp control sequence (5′$A(GA)_3GA_3GA(GA_2)_2GA_4GA3$′) were inserted respectively at the *Hin*dIII or *Eco*RI sites flanking the multiple cloning site (MCS) of bacterial vector pTV118N (available from Takara Shuzo, Japan) resulting in plasmid pTVX118. pTVX118 was cleaved with *Pst*I and *Sca*I (lane 1) in restriction buffer consisting of 50 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl. The purine-rich GT probe [5′biotin-$(G_3T)_2G_2TGTG_4TG_2TG_3TG3$′, 50 pmol] was added to the restriction solution (10 μl, ≈3 μg pTVX118), incubated for one hour at 37°C, and collected by magnetic separation using streptavidin-coated magnetic beads (Dynal Streptavidin M-280, 1 mg suspended in 40 μl of the same buffer). The beads were washed three times with 3 volumes of the same buffer, with (+) or without (−) 0.02% SDS. The triplex complex was disrupted by incubation of the beads in TE buffer (10 mM Tris−HCl, 1 mM EDTA, pH 7.5) at room temperature for 5 min, and the double-stranded DNA was released from the probe (lanes 4, 5). Triplex formation by the homopyrimidine CT probe [5′biotin-$CTC_3TC_2TC_4TCTC_2(TC_3)_23$′] proceeded similarly except that the restriction solution was first ethanol precipitated and then incubated with the probe in acidic buffer (50 mM Tris−acetate, pH 5.4, 50 mM NaCl, 5 mM $MgCl_2$, 0.4 mM spermidine). Captured DNAs were released from the probe (lanes 2, 3) by incubation in alkaline buffer (50 mM Tris−HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$) Lane 2 demonstrates that nonspecific interactions occur with the homopyrimidine probe at low salt conditions in the absence of SDS.

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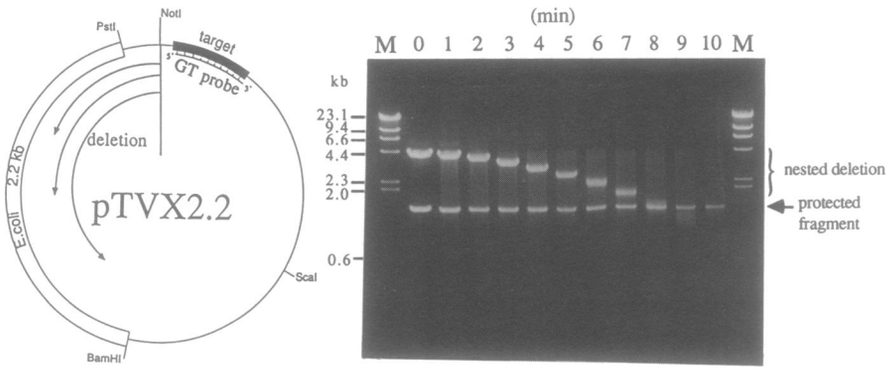

Figure 2. Construction of unidirectional deletions mediated by triplex formation. A 2.2 kb fragment from the *E.coli* genome was inserted between the *Bam*HI and *Pst*I sites of pTVX118 resulting in plasmid pTVX2.2. The plasmid (5 μg) was linearized by *Not*I digestion, resuspended in 50 mM Tris−HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and incubated for 1 hour at 37°C with 125 pmol of 5′-amino purine-rich GT probe (see legend to Figure 1 for probe sequences). The distance between the 3′ end of the *Not*I cleavage site and the 3′ end of the target's purine sequence was 31 bases. Exonuclease III (180 units) was added and the reaction was serially terminated at one minute intervals by the addition of the Mung-bean nuclease cocktail which, at the same time, processed 5′-overhangs generated by Exonuclease III (Deletion Kit for Kilo-Sequencing, Takara Shuzo). DNA samples were purified and digested with *Sca*I, and analyzed by agarose gel electrophoresis.

by washing with alkaline buffer, while the purine-rich probe retains its target until EDTA is added for chelation of Mg^{2+} (data not shown).

Additionally, the stability of the bond between the purine-rich GT probe and the purine strand of the double-stranded target was such that it could block 3′→5′ exonuclease activity of Exonuclease III on a linear DNA substrate, thus permitting the generation of unidirectional deletions. A fragment of unknown sequence originating from the *E.coli* genome was inserted into the triplex vector described above, and the resulting plasmid was linearized with a rare-cutting enzyme *Not*I. The homopurine target sequence was on the strand that was to be processed by Exonuclease III, with its 3′ end positioned 31 bases downstream from one of the 3′-recessed terminals generated by *Not*I cleavage. The preparation was incubated with the purine-rich GT probe and treated directly with Exonuclease III. As seen in Figure 2, the putative triplex formed at the target site was able to effecfively block the progression of exonuclease during the first 7 minutes, while a series of unidirectional deletions were created into the cloned *E.coli* fragment region from the unprotected end. As this example illustrates, the stability and the flexibility of triplex formation with purine-rich GT probes allow an extension in the triplex technology in that it can be used not only for direct separation of DNA from enzyme reaction solutions, but also for selective protection of specific DNA sequences during those manipulations. The frequent occurrence of homopurine tracts in eukaryotic and viral genomes (3, 6) also suggests that this methodology could contribute to the rapid recovery of native DNA-protein complexes which would be important for the study of *in vivo* DNA interactions.

ACKNOWLEDGEMENTS

We thank H.Hara for help in oligonucleotide synthesis, and the members of the Takara laboratories for comments on the manuscript.

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