The stability of different forms of double-stranded decoy DNA in serum and nuclear extracts

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The degradation of single-stranded (ss) oligonucleotides and their analogues in serum and within mammalian cells has been studied extensively because of the potential importance of antisense oligonucleotides as pharmaceutical agents (1–5). Double-stranded (ds) oligonucleotides did not seem to be comparably useful, so little is known about their degradation. Recently, however, it has been shown that ds decoy DNA, that is, DNA containing the specific sequences recognized by transcription factors, can be used to block transcription (6–8) in much the same way that antisense oligonucleotides are used to inactivate RNA once it has been transcribed. Consequently, the stability of ds DNA in serum and inside cells is now of considerable interest.

We have previously shown that transcription factors that bind to standard ds DNA bind with undiminished affinity to equivalent hairpin and dumbbell sequences (9). Since ds sequences in hairpin or dumbbell form cannot undergo strand separation, they should be advantageous as decoys. In this paper we compare the stabilities of each of these forms of ds DNA in human serum and HeLa cell nuclear extracts.

MATERIALS AND METHODS

HeLa cell nuclear extracts were obtained from Promega. Human blood samples, obtained from volunteers at The Salk Institute, were allowed to clot for 30 min and the cells removed by centrifugation.

All oligonucleotides included the CREB transcription factor recognition sequence 5'-TGA CGT CA-3' as a subsequence. The internally labelled 23mer, 5'-AGT TAC CAT GA*pC GTC AAT TTC AG-3', was made by ligating the 11mer 5'-AGT TAC CAT GA-3' to 5'-[³²P]-CGT CAA TTT CAG at 15°C overnight in the presence of a complementary 18mer 5'-AAA TTG ACG TCA TGG TAA-3', and purified by 20% denaturing PAGE. The ds hybrid was made by annealing the internally [³²P]-labelled 23mer to an unlabelled complementary 23mer sequence. Hybridization of the labelled strand was shown to be complete by 20% non-denaturing PAGE. The internally [³²P]-labelled hairpin 41mer was prepared by ligating 5'-[³²P]-GCA GTT AAA-3' to a 32mer 5'-TTT AAC TGC AGT ACC ATT CTC TCA ATG GTA CT-3' and purified by 12% denaturing PAGE. The [³²P]-labelled dumbbell oligonucleotide was synthesized as previously described (9).

Oligonucleotides (2–8 μM) were incubated at 37°C for appropriate times with 90% human serum, or HeLa cell nuclear

extracts (containing 2.1 mg protein/ml) in buffer containing 15 mM Tris, 30 mM KCl, 0.06 mM EDTA and 7% glycerol. Aliquots (2–5 μl) were diluted with phosphate-buffered saline and analyzed on denaturing gels.

RESULTS AND DISCUSSION

Figure 1a illustrates the breakdown in 90% human serum of the ss, ds, hairpin and dumbbell oligonucleotides. The 20% denaturing gel was run for a short time so that the monomer and inorganic phosphate (Pi) are retained on the gel. It indicates that the ss sequence is substantially degraded in 2 h (lane 1). By 4 h it has been almost completely converted to the monomer and Pi (lane 2). By contrast, less than 10% of the ds sequence is degraded, even after overnight incubation at 37°C (lane 5). After 2 h incubation at 37°C, 20% of the hairpin (lane 6) and 5% of the dumbbell (lane 9) are converted to labelled sequences approximately 20–23 bases long. These breakdown products are not degraded rapidly to monomers and Pi, indicating they are present in the serum in the ds form. Only after overnight incubation is a significant percentage of the hairpin (30%) converted to inorganic phosphate (lane 8). At this time less than 5% of the dumbbell has been degraded to give Pi, while 12% of this substrate has been converted to oligonucleotides ~23 bases long (lane 11).

To detect single nicks in the dumbbell sequence, a 4 h sample was analyzed on a 12% denaturing gel (Figure 1b) on which the dumbbell runs considerably faster (lane A) than the unligated dumbbell (lane C). The results shown in lane 1 indicate that a considerable percentage of the dumbbell (40–55%) has been nicked to form linear oligonucleotides ~41–46 bases long. Their slow further degradation to ds 23mers indicate these oligonucleotides are in the hairpin form. Thus, although only 45–60% of the intact dumbbell survives for 4 h in human serum, most of the degraded material is still in a double-helical structure.

Figure 2 shows an autoradiogram of the various oligonucleotides after incubation with a HeLa cell nuclear extract. The ss sequence is substantially broken down within 20 mins (lane 1). After 1 hour 80–90% has been degraded to the monomer and Pi (lane 2). The ds hybrid was also degraded extensively in the nuclear cell extract within 20 mins (lane 3). After 2 h 80% has been degraded (lane 5). Degradation of the hairpin sequence was also detectable after 20 mins (lane 6) and more than 50% was degraded to small M.W. products within 2 h (lane 8). In contrast, the dumbbell remains unchanged after 2 h (lane 11).

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Figure 1. a. Autoradiogram of 20% denaturing gels showing breakdown of internally [^{32}P]-labelled oligodeoxynucleotides in 90% human serum. Lanes 1–2) ss 23mer after 2 and 4 h; lanes 3–5) ds 23mer, after 2 h, 4 h and overnight; lanes 6–8) hairpin 41mer, after 2 h, 4 h and overnight; lanes 9–11) dumbbell 46mer, after 2 h, 4 h and overnight. Lane A) 23mer marker; lane B) 41mer hairpin marker; lane C) dumbbell 46mer marker. b. Autoradiogram of 12% denaturing gel showing: lane 1) formation of nicked dumbbell after 4 h incubation in 90% human serum. Lane A) dumbbell 46mer marker; lane B) 41mer hairpin marker; lane C) unligated dumbbell 46mer marker.

Figure 2. Autoradiogram of a 20% denaturing gel showing breakdown of internally [^{32}P]-labelled ss and ds DNA structures in HeLa cell nuclear extracts at 37°C. Lanes 1–2) ss 23mer after 20 mins and 1 h; lanes 3–5) ds 23mer, after 20 mins, 1 and 2 h; lanes 6–8) hairpin 41mer, after 20 mins, 1 and 2 h; lanes 9–11) dumbbell 46mer after 20 mins, 1 and 2 h. Lane A) 23mer marker; lane B) 41mer hairpin marker; lane C) dumbbell 46mer marker.

Analysis on a 12% gel confirmed that the dumbbell is completely unnicked and undegraded after 2 h (results not shown).

The experiments described above show that in 90% human serum the standard ds DNA remains stable. The ss loops in hairpin and dumbbell DNA are degraded faster than the ds stems. Consequently, hairpin DNA is converted first to ds DNA, while dumbbell DNA is converted first to hairpin DNA and then to ds DNA (Figure 3). The ds DNA subsequently breaks down more

Figure 3. Endonuclease degradation of hairpin and dumbbell DNA. After the initial cleavage of the loop sequence illustrated in the figure, the ends would be nibbled away by exonucleases.

slowly. These results indicate that human sera contain active ss endonucleases and less rapidly acting ds exonucleases. Similar results were obtained for 10% fetal calf serum (results not shown). Very different results are obtained when the oligodeoxynucleotides are incubated with HeLa cell nuclear extracts. The ss, ds and hairpin DNA are all degraded more or less rapidly. However, dumbbell DNA is very stable. It is not significantly changed even after 2 h. The nuclear extract must contain active exonucleases but no endonucleases.

These results illustrate the advantages and the limitations of different forms of ds DNAs as transcription decoys. They show that in nuclear extracts, dumbbell DNA is much more stable than ds or hairpin DNA. However, the nicking of dumbbell DNA in human serum, caused by endonuclease degradation of the ss loops, is a potential problem, since it converts dumbbell DNA to a ds form that is no more stable in the nucleus than standard ds DNA.

Dumbbell DNA should be an ideal transcription decoy for topical application or for delivery in liposomes. DNA should then accumulate in the nucleus without being exposed significantly to endonucleases in the serum. The inclusion of phosphorothioate groups in the ss loops of dumbbell DNA could make it very stable to degradation in human serum, without affecting its ability to decoy transcription factors.

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