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SEQUENCE-SPECIFIC DNA BREAKS PRODUCED BY TRIPLEX-DIRECTED DECAY OF IODINE-125

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Triplex forming oligonucleotides (TFO) labeled with Auger emitters could be ideal vehicles to deliver radioactive-decay energy to specific DNA sequences, causing DNA breaks and, subsequently, inactivation of these sequences. To demonstrate this approach we labeled with ^{125}I (two ^{125}I per molecule on average) a purine-rich 38-mer which forms a stable triplex with a polypurine · polypyrimidine stretch in the human HPRT gene. Decay of ^{125}I in the bound TFO was shown to cause sequence-specific double strand breaks (DSB) in the target HPRT sequence cloned into plasmid DNA. No sequence-specific breaks were observed if ^{125}I-labeled TFO were not bound to the plasmid DNA. After 60 days of decay accumulation (one ^{125}I half-life) approximately a quarter of all plasmid molecules contained sequence-specific DSB, corresponding to 0.3 site-specific DSB per decay. Sequencing gel analysis shows that the DNA breaks are distributed within a few bases of the maxima at those bases opposite to the positions of ^{125}I in the TFO.

Therapeutic applications of Auger electron emitters depend upon developing methods for radionuclide delivery to the nucleus of target cells, for example cancer cells or virally infected cells. The ideal vector for delivering Auger electron emitters should specifically accumulate in the targeted cells and concentrate in the cell nucleus in close proximity to the DNA. Decay of the radionuclide would deliver a high dose of radiation to these cells, introducing highly toxic double-strand breaks (DSB) in their genomes, while nearby cells would be exposed to substantially lesser doses (reviewed in (1)). A level of specificity could be introduced if the delivery vector targets a specific sequence within the genome, for example a sequence that appeared as the result of genomic rearrangements often associated with cancer (2) or a proviral genome in the case of viral infection. In addition, targeting of DSB to specific sequences in DNA could find a use in a wide range of applications, from genome mapping to gene therapy.

In recent years numerous studies on DNA sequence-specific actions were concentrated on using short oligonucleotides, 20 to 50 bases long, that can form triple helixes with particular DNA sequences; so-called triplex-forming oligonucleotides (TFO) (reviewed in (3)). In a triplex, TFO occupies the major grove of the DNA double helix-forming Hoogsteen pairs with the purines (Pu) of the Watson-Crick basepairs and, therefore, directly reading the sequence information of the DNA. In general, stable triplexes can be formed between polypurine·polypyrimidine (Pu · Py) duplexes and polypurine or polypyrimidine oligonucleotides. Such sequences are widespread in eukariotic genomes and are often found in important regulatory regions. It has been shown that TFO are capable of blocking transcription of specific genes containing their target sequences (4). We have shown that TFO can serve as suitable vehicles to deliver iodine-125 to a specific site in DNA (5). Thus, ^{125}I-labeled pyrimidine-rich TFO targeted against a Py · Pu tract in DNA produced DSB specifically in the target sequence. The efficiency of DSB per decay was close to 0.8; however, the overall efficiency was low, only 4% of DNA molecules were cleaved after 56 days of

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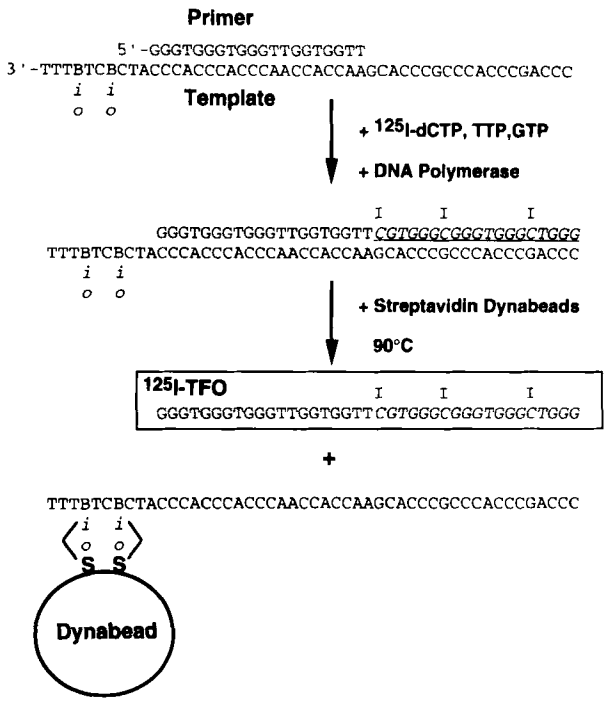


Fig. 1. Synthesis of ^{125}I labeled TFO. Two synthetic oligonucleotides, 20-mer (primer) and 49-mer (template) were annealed and the 3' end of the primer were filled in by DNA polymerase I (Klenow fragment) in the presence of ^{125}I-dCTP, TTP and dGTP. The ^{125}I-TFO were purified by denaturing the resulting duplex at 90°C followed by precipitation of the biotinylated template with streptavidin magnetic beads (Dynabeads).

decay accumulation mainly due to low (less than 10%) incorporation of ^{125}I-dCTP into the TFO.

Here we present data that show ^{125}I-TFO-induced DSB using TFO with high specific activity due to increased incorporation via multiple-site labeling. We also extend our study to another type of triplex, which is stabilized by G · G · C and T · A · T triads and is stable at physiological conditions. Higher specific activity TFO allowed us to observe DSB using as a target for cleavage whole plasmid DNA and even total human genomic DNA. We also show that despite the fact that ^{125}I-dCTP bases were in mismatch positions in the triplex, decay of the radioiodines still produces DSB.

Material and Methods

DNA. A fragment of HPRT gene was amplified by PCR using primers at positions 11 967 and 12 799 of HPRT gene sequence (6). As a substrate for PCR we used Huλ3 clone of human genomic library (ATCC No. 57 238). The PCR fragment was cloned into pCR3 vector (Invitrogene) following manufacturer's instructions. The resulting plasmid pCR3HPRT was purified from E. coli using Maxiprep kit (Qiagene) and the sequence of the fragment was confirmed by dideoxy sequencing (Sequenase 2, USB). Prior to use, supercoiled plasmid was relaxed with topoisomerase I (Gibco-BRL). Genomic DNA from HeLa and HT1080 cells was purified with Nucleon II kit (Scotlab).

Oligonucleotides. Oligonucleotides were synthesized on an ABI-394 DNA synthesizer (Applied Biosystems) followed by purification from polyacrylamide gel (PAG). Template oligonucleotide was biotinylated using BioTEG modifiers (Glen Research). Sequences of the template oligonucleotide and primer oligonucleotide for TFO-HPRT are shown in Fig. 1. Primer and template oligonucleotides (typically 10 pmol each) were annealed in 1xPol buffer (10 mM Tris · HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DDT) 5 min incubation at 65°C followed by slow cooling to room temperature. Primer extension reaction was carried out in the presence of 1 mM of dATP, TTP, dGTP and 100 μCi of lyophilized ^{125}I-dCTP (DuPont-NEN) by 0.5 unit of exonuclease-free Klenow fragment of DNA polymerase I (USB) in a total volume of 10 μl at room temperature. After 10 min, reaction was stopped by 30 μl 10 mM EDTA, extracted with phenol:chloroform and the product duplex was purified by gel filtration through a G-50 micro column (Pharmacia) equilibrated with TES buffer (50 mM Tris · HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl). At this step the amount of incorporated ^{125}I-dC was calculated. The labeled duplex was bound to Streptavidin Dynabeads (Dynal) by incubation for 30 min at room temperature in TES buffer. Beads were collected with a magnet and resuspended in water. The duplex was denatured by heating at 80°C for 1 min and beads with bound template oligonucleotide were immediately collected on ice. Supernatant containing ^{125}I-TFO was further purified by gel filtration into TE buffer (50 mM Tris · HCl, pH 7.5, 1 mM EDTA) and stored at −70°C.

Binding. Four microcuries of ^{125}I-TFO (approximately 0.9 pmol) were mixed with 0.6 pmol of pCR3HPRT plasmid or with 20 μg genomic DNA in conditions indicated in the text. For triplex formation the mixtures were incubated at 65°C for 5 min and slowly cooled down to room temperature. Samples were aliquoted and kept at −70°C for accumulation of decays. Aliquots of the samples were analyzed by 2% Agarose gel electrophoresis at 10°C using 90 mM Tris-borate, pH 8.3, containing 3 mM $MgCl_2$ as an electrode buffer for 3 h at 5 volts/cm. Triplex formation was detected by band shift. Gels were fixed in 10% Trichloroacetic acid, dried and visualized with a BAS 1500 Bio-Imaging Analyzer (Fuji).

Breaks analysis

Aliquots of the samples were thawed after the indicated time and then were electrophoresed through 1% Agarose gel containing 0.5 μg/ml ethidium bromide. The remaining samples were cut with restriction enzymes (indicated in the

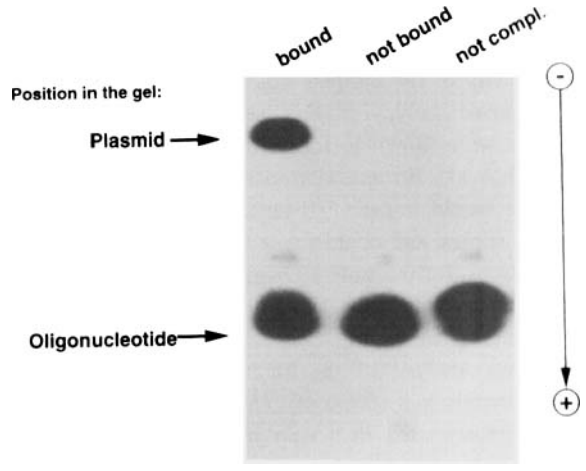


Fig. 2. Binding of oligonucleotides to plasmid DNA. Covalently closed, relaxed DNA of pCR3HPRT was preincubated with ^{125}I-HPRT oligonucleotide in TMSp (binding) or in TE (not binding) buffers, or with ^{125}I-O oligonucleotide in TMSp buffer (not compl.). The samples were separated in 2% Agarose gel. Positions of free oligonucleotides and plasmid bands along with direction of electrophoresis are shown.

text) for further analysis. PvuI-cut plasmid were analyzed by electrophoresis in 1% Agarose. Agarose gels were stained with ethidium bromide and quantitated with a CCD camera connected to a Macintosh computer using the NIH image software.

Where indicated, plasmid was labeled at the 5′ end by T4 polynucleotide kinase (Gibco-BRL) and ^{32}P-γATP, purified with phenol:chloroform extraction followed by gel filtration and cut with a second restriction enzyme. Labeled DNA fragments were separated in native and denaturing polyacrylamide gels as described (5). Gels were fixed in 10% acetic acid and quantitated with a BAS 1500 Bio-Imaging Analyzer (Fuji).

Genomic DNA was electrophoresed through 1% Agarose gel and transferred to a Genescreen hybridization membrane (DuPont-NEN). Plasmid DNA was cut with PflMI and KpnI restriction enzymes and a small fragment was purified from an Agarose gel. The fragment was labeled with Oligolabeling kit (Pharmacia), hybridized with the membrane following manufacturer's instruction and visualized with a BAS 1500 Bio-Imaging Analyzer (Fuji).

Results

Labeling of TFO with ^{125}I. As shown in Fig. 1 TFO used in the present study could potentially contain three ^{125}I-dC's. We noticed that ^{125}I-dCTP is incorporated by DNA polymerases considerably poorer than ^{32}P-dCTP. Therefore, the reaction was carried out at the highest possible ^{125}I-dCTP concentration for a longer period of time or at higher enzyme concentration using exonuclease-free

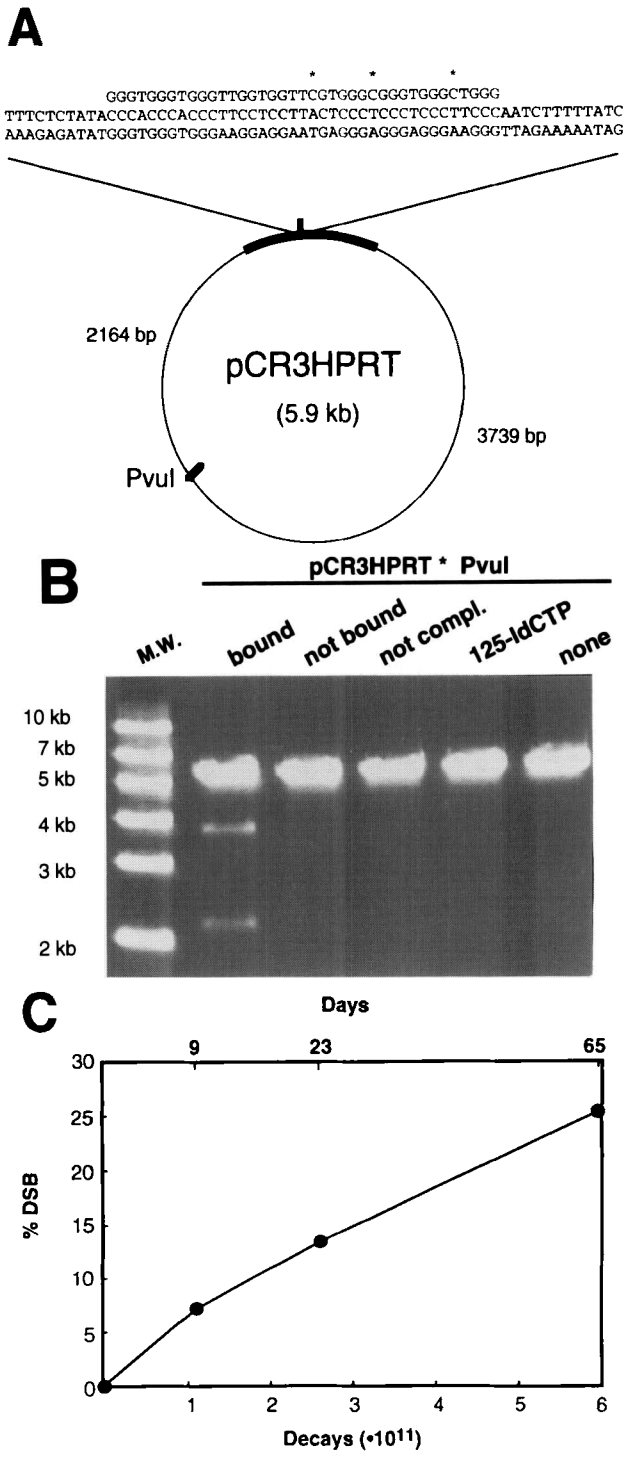


Fig. 3. Double strand breaks in plasmid DNA. A. The scheme of the pCR3HPRT plasmid. The 833 bp PCR fragment cloned into pCR3 vector is shown in bold. Inset shows the target sequence along with TFO. Possible positions of ^{125}I dC's are marked with stars. Distances in bp from the target sequence to the single PvuI site are also shown. B. 1% Agarose gel of the samples of pCR3HPRT cut with PvuI after nine days of decay accumulation (corresponds to 0.5 decay per plasmid). The type of oligonucleotide and conditions in the samples are shown at the top of the lanes. The size of molecular weight markers (M.W.) is shown on the left. C. The percentage of DSB as a function of time and accumulated decays.

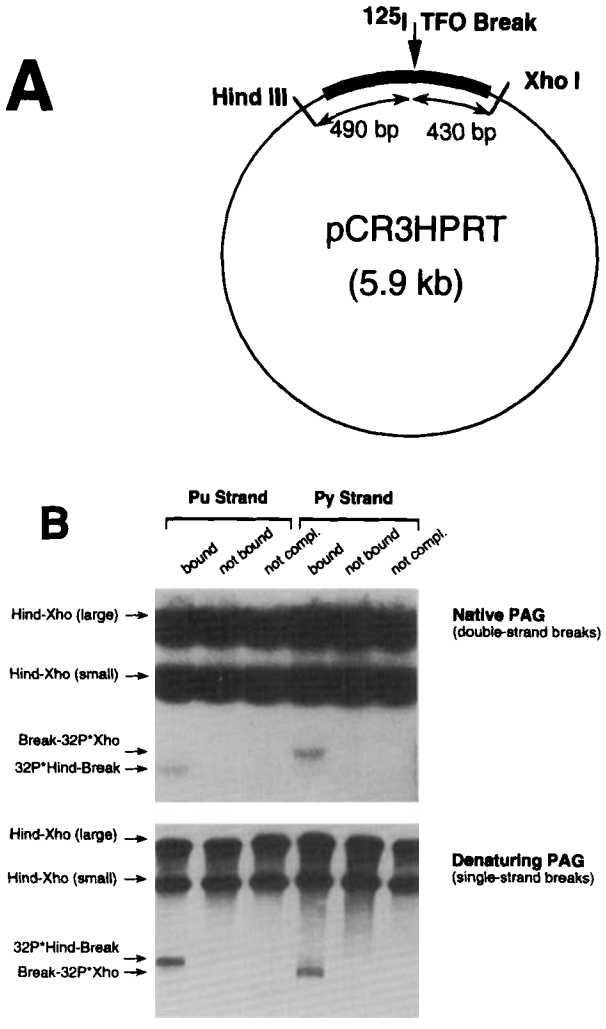


Fig. 4. Double and single strand breaks in plasmid DNA. A. The scheme of the pCR3HPRT plasmid. Positions of the target sequence, HindIII and XhoI restriction enzymes cleavage sites and distances between them are shown. B. Autoradiographs of native and denaturing 10% polyacrylamide gels. After nine days of decay accumulation samples were cut with HindIII and XhoI restriction enzymes and labeled at Pu (HindIII) or Py (XhoI) strand. Positions of the resulting fragments in the gel are shown on the left. The type of oligonucleotide and conditions in the samples are shown at the top of the lanes.

Klenow fragment of DNA polymerase I (see Material and Methods). The incorporation varied from batch to batch of ^{125}I-dCTP used and at maximum reached two ^{125}I-dC per TFO.

Binding of TFO to plasmid. Binding of TFO to pCR3HPRT was carried out in 50 mM Tris · HCl, pH 8, 10 mM $MgCl_2$ and 1 mM Spermidine (TMSp buffer) at 65°C for 5 min followed by slow cooling to room temperature (bound). As a control we incubated the plasmid with ^{125}I-TFO in TE buffer where no binding was expected (not bound). We also incubated plasmid under binding conditions with ^{125}I-labeled oligonucleotide (^{125}I-O) with a scrabbled sequence that could not form a triplex (not complementary) and with ^{125}I-dCTP alone. In all cases the radioactivity of the samples was approximately the same.

Polypurine · polypyrimidine sequences in supercoiled plasmids were shown to form alternative structures, such as H-DNA (7). Structural transitions in a Pu · Py target sequence would impede ^{125}I-labeled oligonucleotide from forming triplex and could favor formation of other types of structures, for example a D-loop. To prevent the supercoiling-driven transitions, substrate plasmid was relaxed by topoisomerase I.

Gel-shift analysis of the binding is shown in Fig. 2. Approximately half (55%) of ^{125}I-TFO bound to the plasmid if preincubated in TMSp buffer (see above) which appeared as a band comigrating with the plasmid ('bound'). As expected, no binding occurred in the 'not bound' and 'not complementary' controls.

Site-specific DSB. Aliquots of the samples were cut with PvuI restriction enzyme which has the only site of cleavage in pCR3HPRT plasmid (Fig. 3A) and analyzed on 1% Agarose gel. In the case of the plasmid that has been frozen with bound ^{125}I-TFO ('bound') two fragments appear in the gel (Fig. 3B). The length of the fragments, 2.2 kbp and 3.7 kbp, corresponds to DSB in the target sequence. No sequence-specific breaks were observed if ^{125}I-TFO was not bound to the target sequence ('not bound', 'not complementary' and '^{125}I-dCTP' controls). The percentage of the breaks was calculated as the ratio of the sum of the intensities of the 2.2 kbp and 3.7 kbp fragments to the sum of the intensities of all three fragments in the lane. The results for different times of decay accumulation are presented on Fig. 3C.

Double- vs. single strand site-specific breaks. After accumulation of decays plasmid DNA was cut with HindIII and XhoI restriction enzymes (Fig. 4A). The 5′ overhangs produced by these enzymes are AGCT and TCGA correspondingly. Therefore, the HindIII end can be selectively labeled with ^{32}P-dATP while the XhoI end can be labeled with ^{32}P-dCTP and TTP. In the fragment labeled at the HindIII site the purine (Pu) strand of the target polypurine · polypurimidine sequence is labeled. For the XhoI labeled fragment the pyrimidine (Py) strand is labeled. Labeled samples were analyzed in native PAG to measure DSB and in denaturing PAG to measure total SSB (Fig. 4B). In the sample with bound ^{125}I-TFO shorter fragments appear in both Pu and Py labeled strands. The length of the fragments in a denaturing gel corresponds to the expected position of ^{125}I-TFO. In native gel XhoI labeled fragment migrates somewhat slower than expected. The anomalous mobility of the fragments containing repeated sequences has been observed before and is most likely caused by bending (8). No site-specific breaks were observed in any of the controls. The amount of breaks was

Table

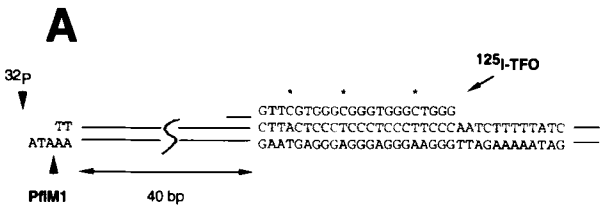
Amount of double and single strand breaks measured by end Labeling assay

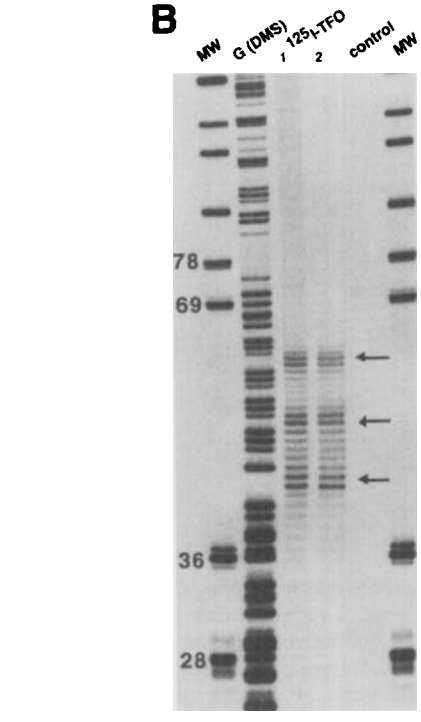
	9 days DSB (%)	SSB (%)	23 days DSB (%)	SSB (%)
Pu strand	6	11	10	23
Py strand	6	8	11	18

measured as described before (5). The results are summarized in the Table . For all doses of radiation the amount of SSB is consistently higher than DSB. The amount of SSB in Pu strand in higher than in Py strand.

Distribution of the breaks at the nucleotide level. After 47 days of decay accumulation, which corresponds to two decays per molecule of plasmid, aliquots of plasmid DNA were cut with PflM1 restriction enzyme which has a cleavage site 40 bp upstream of the target sequence (Fig. 5A) and labeled at the 3′ end with terminal deoxinucleotidyl transferase and 2′-^{32}P-ddATP (Fig 5B, lane ^{125}I-TFO 2). To generate a G sequencing ladder an aliquot of labeled native plasmid was treated with dimethylsulfate followed by digestion in hot piperidine (9) (lane G(DMS)). To test the possibility of alkali-liable modification an aliquot of the sample that had accumulated decays with the ^{125}I-TFO bound was also treated with hot piperidine (Fig 5B, lane ^{125}I-TFO 1). Decay of ^{125}I in the TFO causes breaks in the Pu strand of the target duplex that are distributed in the narrow region around the sites of ^{125}I-dC in TFO with three maxima exactly in front of these sites. The distribution proves that ^{125}I-dCTP was incorporated in all three potential sites on TFO with approximately equal probability. Treatment with hot piperidine did not result in any significant changes in distribution and intensity of the breaks. Therefore, the decay of ^{125}I in close proximity to DNA causes predominantly direct strand breaks.

Site-specific breaks in genomic DNA. To prove that the target sequence is present in HeLa and HT1080 cells the fragment of their genome was PCR-amplified using the same primers we used for construction of pCR3HPRT plasmid (see Material and Methods). The fragments were subcloned and the presence of the target sequence was proved by direct sequencing (data not shown). Genomic DNA purified from HeLa and HT1080 cells were cut with PstI restriction enzyme and separated in 1% Agarose gel. DNA was transferred from the gel to a hybridization membrane and probed with 420 bp PflMI-KpnI fragment of pCR3HPRT (Fig. 6). The target sequence is located in 2.4 kb PstI fragment (Fig. 6A). In the samples that had been frozen with ^{125}I-TFO bound, a 1 kb band appears that corresponds to DSB in the target sequence (Fig. 6B). Since only 40 bp of the probe were homologous to the 1.4 kb





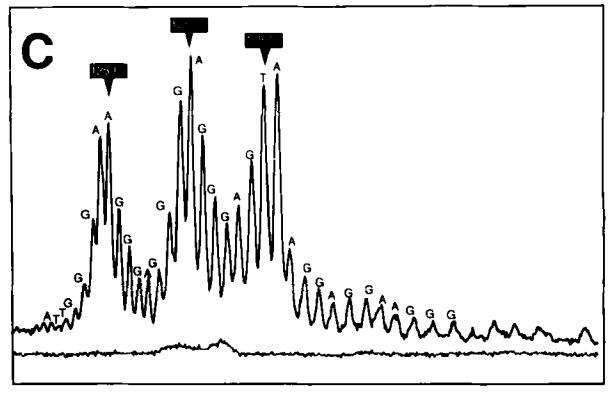


Fig. 5. Analysis of the breaks on the sequencing level. A. The scheme of the target sequence, TFO and PflM1 restriction enzyme site. B. Autoradiograph of 12% polyacrylamide gel. MW-molecular weight markers; ^{32}P labeled DNA pUC18 cut with MspI restriction enzyme. Length of some fragments in nucleotides is shown on the left. pCR3HPRT was cut with PflM1 and labeled at the 3′ end with ^{32}P. G(DMS)-Maxam-Gilbert G-ladder. ^{125}I-TFO-samples after 47 days of decay accumulation. Sample 1 was treated with hot piperidine. Control-native pCR3HPRT sample. C. Densitogramm of the lanes ^{125}I-TFO (2) and control from panel B. Corresponding bases are shown at the top of the peaks; arrows mark the positions of ^{125}I-dC's in TFO.

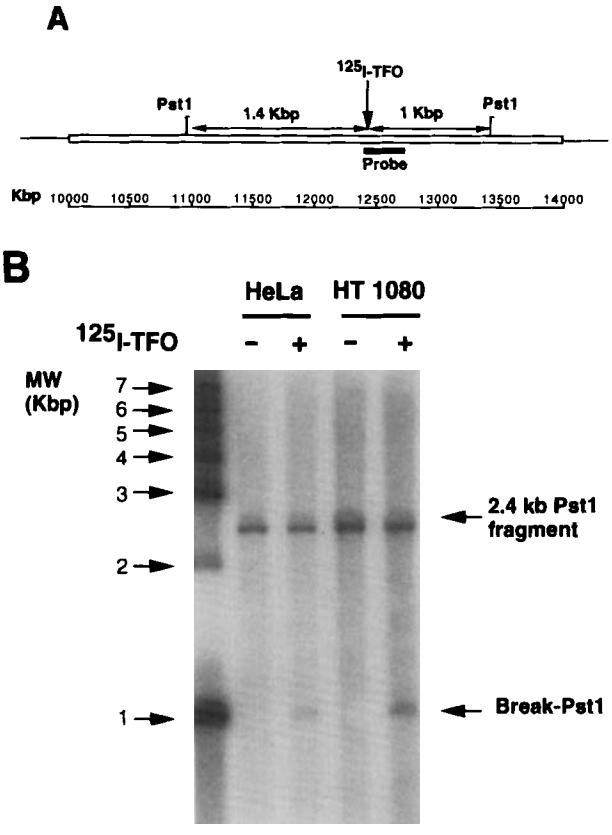


Fig. 6. ^{125}I-TFO induced breaks in genomic DNA. A. The target region in the HPRT gene. Two consecutive Pst1 restriction enzyme sites, position of the TFO binding site and the probe are shown. Distances in kilobasepairs along the HPRT sequenced region are shown on the bottom. B. Southern blot analysis of Pst1 digested genomic DNA from HeLa and HT1080 cells. Molecular weight markers are shown on the left. Lanes marked with "+" correspond to the samples incubated with ^{125}I-TFO.

fragment it could not be detected under the hybridization conditions. Quantitation of the radioautograph showed that 25% of genomic DNA received DSB after 45 days of decay accumulation which corresponds to ca. 10^5 decays per genome.

Discussion

The data presented in this paper show that ^{125}I-TFO labeled to high specific activity efficiently produce breaks in a target sequence upon Pu · Pu · Py type triplex formation. After 65 days of decay accumulation which corresponds to 2.6 decays per plasmid 25% of plasmid DNA molecules were cleaved (Fig. 3C). The efficiency in terms of DSB per decay is lower than we observed for Py · Pu · Py triplex (5). Indeed, if each decay produces one DSB then after 65 days of decay accumulation which is close to one half-life for ^{125}I one would expect 50% yield for DSB. Taking into account that each TFO has two ^{125}I atoms on average and assuming that the iodines distributed evenly among TFO one can estimate that 75% of the molecules would be cleaved. This means that every third decay produces a DSB.

This is a very rough estimation, since at this stage we do not know several experimental parameters. First, we do not know how in fact the ^{125}I atoms are distributed among TFO. If, for example, 66% of TFO have 3 ^{125}I-dC and 34% are not labeled at all, then our estimation for the efficiency would be higher than 0.3 DSB per decay. Second, we cannot exclude that some radioiodines could have been lost during purification procedure which would reduce the specific activity of the TFO. Third, the G-rich TFO is known for forming intra- and intermolecular structures which prevent triplex formation with the target sequence. In this case some of the targets might not be occupied even if we observed saturation in our binding assay (Fig. 2) which would result in an underestimation of the amount of DSB per decay.

But there are principal reasons for lower efficiency per decay. The TFO used here binds to the target duplex via G · G · C and T · A · T triads and, therefore, the dC's are in the mismatch positions and, presumably, do not form hydrogen bonds with the target (Fig. 3A and (3)). This means, that ^{125}I is located further away from the double helix than in the case of duplex or Py · Pu · Py protonated triplex, and thus its decay is less efficient in the production of DSB. This notion is also supported by the fact that there are two times more SSB then DSB in the target sequence and that the amount of SSB differs for Pu and Py strands (Table). The difference in ionic conditions during accumulation of decays for Py · Pu · Py and Pu · Pu · Py triplexes could also affect the yield of DSB per decay.

The sequence-specific cleavage of total genomic DNA (Fig. 6) is the most impressive demonstration of the ability of ^{125}I-TFO to target specific sites in a genome. The next step will be to show sequence-specific breaks in the HPRT locus in cells treated with ^{125}I-TFO. In addition to a DNA-breaks analysis, radiation damage in the HPRT gene could be detected with high sensitivity by mutant selection (10). Further work is currently in progress.

REFERENCES

1. Sastry KS. Biological effects of the Auger emitter iodine-125: a review. Report No. 1 of AAPM Nuclear Medicine Task Group No. 6 (see comments). Med Phys 1992; 19: 1361–70.
2. Bouffler S, Silver A, Cox R. The role of DNA repeats and associated secondary structures in genomic instability and neoplasia. BioEssays 1993; 15: 409–12.
3. Frank-Kamenetskii MD, Mirkin SM. Triplex DNA structures. Annu Rev Biochem 1995; 64: 64–95.
4. Helene C, Thuong NT, Harel-Bellan A. Control of gene expression by triple helix-forming oligonucleotides. The antigene strategy. Ann N Y Acad Sci USA 1992; 660: 27–36.
5. Panyutin IG, Neumann RD. Sequence-specific DNA double-strand breaks induced by triplex forming ^{125}I labeled oligonucleotides. Nucleic Acids Res 1994; 22: 4979–82.

6. Edwards A, Voss H, Rice P, et al. Automated DNA sequencing of the human HPRT locus. Genomics 1990; 6: 593–608.
7. Mirkin SM, Frank-Kamenetskii MD. H-DNA and related structures. Annu Rev Biophys Biomol Struct 1994; 23: 541–76.
8. Hagerman PJ. Flexibility of DNA. Annu Rev. Biophys Biophys Chem 1988; 17: 265–86.
9. Maxam AM, Gilbert W. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol 1980; 65: 499–525.
10. Gibbs RA, Camakaris J, Hodgson GS, Martin RF. Molecular characterization of ^{125}I decay and X-ray-induced HPRT mutants in CHO cells. Int J Radiat Biol Relat Stud Phys Chem Med 1987; 51: 193–9.