Quantitative Assessment of Chimeraplast Stability in Biological Fluids by Polyacrylamide Gel Electrophoresis and Laser-Assisted Fluorescence Analysis

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INTRODUCTION

Synthetic oligonucleotides are becoming very important tools in biomedicine. Recent advances in oligonucleotide chemistry enable remodeling of their structural units for a wide variety of applications, ranging from basic research to diagnosis and therapy (1). In the therapeutic area, antisense oligonucleotides and ribozymes were the first to be developed for inhibition of gene expression at the transcriptional level, which led to the initiation of clinical trials (2,3). Recently, an antisense oligonucleotide has been approved for the treatment of human cytomegalovirus-induced retinitis (4). Antigene strategies using triplex-forming oligonucleotides (TFO) are also becoming useful alternatives to block sequence-specific transcription (5). Moreover, in recent years several oligonucleotide-mediated gene targeting approaches have been envisioned with the purpose of *in situ* mutation correction on genomic DNA (6). One of these approaches is based on the use of hybrid RNA/DNA oligonucleotides, termed "chimeraplasts." These molecules are capable of recognizing and reverting both point and frameshift mutations in cells and animal and plant models by induction of their endogenous mismatch repair mechanisms. Thus, preliminary assays have indicated the huge therapeutic potential of chimeraplasts in the treatment of genetic defects (for a review, see Ref. 7).

A necessary step for the success of oligonucleotide-based therapeutics is to maintain oligonucleotide integrity as much as possible to preserve their function until the target is reached. To increase performance, a variety of modifications have been introduced to the different bases, the sugar backbone, or the internucleoside linkages forming the oligonucleotide chain (1). Thus, phosphorothioate linkages and 2′-*O*-methyl-oligoribonucleotides seem to avoid extra- and intracellular oligonucleotide degradation. Although some of these analogs (e.g., all-phosphorothioate or -methylphosphonate backbones) have considerably improved oligonucleotide stability in a variety of biological fluids, other issues such as poor binding affinity, poor solubility, or deficient penetration through membranes has hampered their performance. Therefore, it is becoming clear that either novel synthetic oligomers such as N3′→P5′ phosphoramidates (8) or combinations of the above modifications, termed "mixed backbone oligonucleotides" (MBOs) (9), are needed to enhance oligonucleotide effectiveness for multiple applications.

Metabolism is an essential biochemical process that needs to be evaluated on new oligonucleotide structures to optimize their performance. Because most of the currently modified oligonucleotides are charged molecules, electrophoretic techniques such as polyacrylamide gel electrophoresis (PAGE) on slab gels or, more recently, the more sophisticated and expensive capillary gel electrophoresis (CGE), are commonly used to assess their turnover in biological fluids (1). For detection and analysis, the method of choice usually includes autoradiography of radioactively labeled oligonucleotides. But radioactivity-based methodologies, although quite sensitive, are cumbersome, time-consuming, and require both trained personnel and specific radioactive installations.

We have developed an easy, fast, safe, and reliable nonradioactive method to assess the pattern and to quantify the percentage of oligonucleotide degradation in biological fluids using fluorescent oligonucleotides. The whole procedure can be easily performed either in a small laboratory, if adequate genotyping instrumentation and analysis software is available, or the sample analysis step may be completed in any automatic sequencing and genotyping facility. We have applied this methodology to quantify the effect of serum on chimeraplast degradation.

MATERIALS AND METHODS

Chimeraplast Synthesis and Purification

CFO-1 is a 68-mer RNA/DNA chimeric oligonucleotide (5′-FITC-TCTGCTAGGACCCCCCACACTTGTTTTT-TaacaagugugGGGGGuccuagcagaGCGCGTTTTCGCGC-3′, where DNA residues are capitalized and 2′-*O*-methyl RNA residues are in lower case). This chimeraplast was synthesized on an 0.2-μmol scale by using the 1000-Å-wide-pore CPG on an ABI 392 DNA/RNA synthesizer (Perkin Elmer, Foster City, California) by standard phosphoramidite procedure. The coupling time was increased to 5 min for the 2′-*O*-methyl RNA phosphoramidites (Cruachem Ltd., Glasgow, Scotland). Fluorescein phosphoramidite was incorporated at the 5′-end with a coupling time of 5 min. After the synthesis was complete, the product was heated in ethanol/concentrated ammonium hydroxide, 1:3 (vol/vol) for 20 h at 55°C to remove the base-protecting groups. Finally, the fluorescent chimeraplast was PAGE purified as previously described (10). Briefly, crude CFO-1 was resuspended in 7 M urea/10% (vol/vol) glycerol, heated at 70°C, and fractionated on a 15% polyacrylamide/7 M urea gel. The 68-mer band was visualized by UV shadowing over a TLC plate (Merck, Darmstadt, Germany) and sliced out from the gel. Gel slices containing the full-length chimeraplast were crushed and incubated overnight in TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 7.5) to release the oligonucleotide from the polymer. Finally, the chimeraplast-containing solution was separated

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from the gel pieces by centrifugation through a 0.45-μm spin-filter (Millipore, Bedford, Massachusetts) and desalted through a Sephadex G-25 column (Amersham Pharmacia Biotech, Uppsala, Sweden), lyophylized, and resuspended in 100 μl of ultrapure water.

Chimeraplast Stability Assays

The stability of CFO-1 (260 nM) was assessed after 24-h and 48-h incubation of the chimeraplast, either naked or complexed with nonviral vectors, in 500-μl LHC-8 medium (Biofluids, Rockville, Maryland), without or with 5% fetal calf serum (FCS). The sources of serum tested were either fresh, stored for 1 month at 4°C with daily warming at 37°C during 1 h (old), or incubated at 56°C for 1 h (inactivated). The incubations were terminated at the indicated times by performing two rounds of phenol/isoamyl alcohol/chloroform (24:1:24) organic extraction of the culture media.

CFO-1/PEI polyplexes were prepared by diluting both the CFO-1 oligonucleotide and ExGen 500 (22 kDa linear polyethylenimine [PEI]; MBI Fermentas, Vilnius, Lithuania) at a 9:1 PEI amine/oligonucleotide phosphate molar ratio (N/P = 9) with 5% dextrose in separate polystyrene tubes up to a volume of 50 μl each. Complexes of an estimated size of 60–100 nm, determined by dynamic light scattering, were formed after adding the PEI vector solution dropwise to the CFO-1 oligonucleotide solution, immediate gentle vortexing to mix, and further incubation for 10 min at room temperature. Generation of CFO-1/cytofectin lipoplexes (estimated size of 200–400 nm; N/P ratio of 0.12) was identical to that for the polyplexes, except that both the CFO-1 chimeraplast and 2.5 μg/ml Cytofectin GSV (GS3815/DOPE; 2:1 molar ratio, Glen Research, Sterling, Virginia) were diluted in LHC-8 basal medium previous to complex formation. The ratios of both chimeraplast/PEI and chimeraplast/cytofectin complexes had been previously optimized in our laboratory for efficient transfection of different cell lines (unpublished results).

Polyacrylamide Gel Electrophoresis and GeneScan Analysis

A 1:5 aqueous dilution of the deproteinized samples was fractionated directly by slab gel electrophoresis under denaturing conditions (5% polyacrylamide/6 M urea gel) on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, California). The fluorescent oligonucleotide metabolites separated on the gel were analyzed and automatically quantified (each band as peak height and peak area) by GeneScan software, v. 2.1 (Applied Biosystems).

RESULTS AND DISCUSSION

We have developed an easy, fast, safe, and sensitive method to assess oligomer metabolism in biological fluids using fluorescent oligonucleotides. Previous studies seemed to indicate a high resistance of the chimeraplasts to nucleolytic hydrolysis due to their structural features, including two RNA domains (10-mer) of 2′-*O*-methyl-protected ribonucleotides and two T-hairpin loops (10) (Fig. 1A). Serum is an important factor affecting oligonucleotide stability in both *in vitro* experiments and *in vivo* assays requiring systemic administration. Therefore, we have used our fluorescent oligonucleotide-based approach to further analyze the influence of the serum included in the culture medium on the metabolism of a typical chimeraplast, administered either naked or complexed with cationic lipid vesicles (cytofectin) or with the polycation polyethylenimine (PEI). Figure 1B shows the fluorescent degradation patterns of CFO-1 from different samples loaded simultaneously on the denaturing gel. Of note, the excellent resolution of laser-mediated fluorescence detection combined with the fractionation power of PAGE enables single-nucleotide resolution of degradation fragments. Moreover, it should also be possible to detect concatamers formed by end-to-end ligation of chimeraplasts or other oligonucleotides by nondenaturing slab gel electrophoresis using this method. We have determined by dilution experiments that the detection limit of our technique for a reliable oligonucleotide degradation analysis can go down to 10 pM (data not shown). This sensitivity enables its successful application in a variety of *in vitro* and *in vivo* stability experiments. Single-lane automatic quantification of nucleic acid fragment amount by GeneScan software is shown in Fig. 2. The result from electrophoretograms A, B, and C indicates a significant metabolic activity over the CFO-1 chimeraplast after 24 h incubation in LHC-8 culture medium containing 5% fetal calf serum, which increased over time (Table I). Because the LHC-8 human epithelial cell culture medium alone was inert on CFO-1 under the same incubation conditions (electrophoretogram D), the degradation patterns generated must be attributed to the serum present in the medium. This was not surprising, as it is known that ubiquitous nucleases are present in significant amounts in different sources of serum (11). A detailed examination of the analytical profile resulting from incubation of CFO-1 in medium containing fresh serum yielded insight about its metabolism (Fig. 2A). The main right-hand peak represents full-length CFO-1 whereas the immediate metabolite ladder (processive n-1 shortmers) indicates the presence in the serum of 3′-exonucleolytic activity. The corresponding end product is a 54-mer fluorescent oligonucleotide metabolite that lacks the "GC" clamp and one of the T-hairpin loops as compared with the full-length chimeraplast. Another feature of the CFO-1 degradation pattern was the absence of degradation peaks in an intermediate region of about 25 nucleotides. This nuclease-resistant region coincides with the strand of CFO-1 holding the two 10-mer 2′-*O*-methyl-RNA domains and the intermediate 5-mer mutator region (Fig. 1A). It has been shown that oligonucleotides bearing alkyl modifications in the 2′-position of the β-D-ribofuranosyl moiety are strongly protected against nuclease degradation (9). Simultaneous study of the activity patterns corresponding to 5′- and 3′-exonucleases should also be possible by labeling both ends of the chimeraplast molecule with two different fluorescent dyes of nonoverlapping spectra.

Additionally, the electrophoretogram shown in Fig. 2A revealed the appearance of metabolite peaks that migrated earlier, resulting from cleavage after the 2′-*O*-methyl-RNA protected region. This discontinuous pattern of metabolite production appeared to be the result of additional CFO-1 degradation by endonucleolytic activity. It has been suggested that modifying oligonucleotide chemistry may also alter the types of nucleolytic activity involved in oligonucleotide metabolism (12). Concomitant with the loss of full-length CFO-1, shortmers were subjected to additional degradation in a time-dependent fashion (data not shown). Moreover, both thermal treatment for complement inactivation and improper

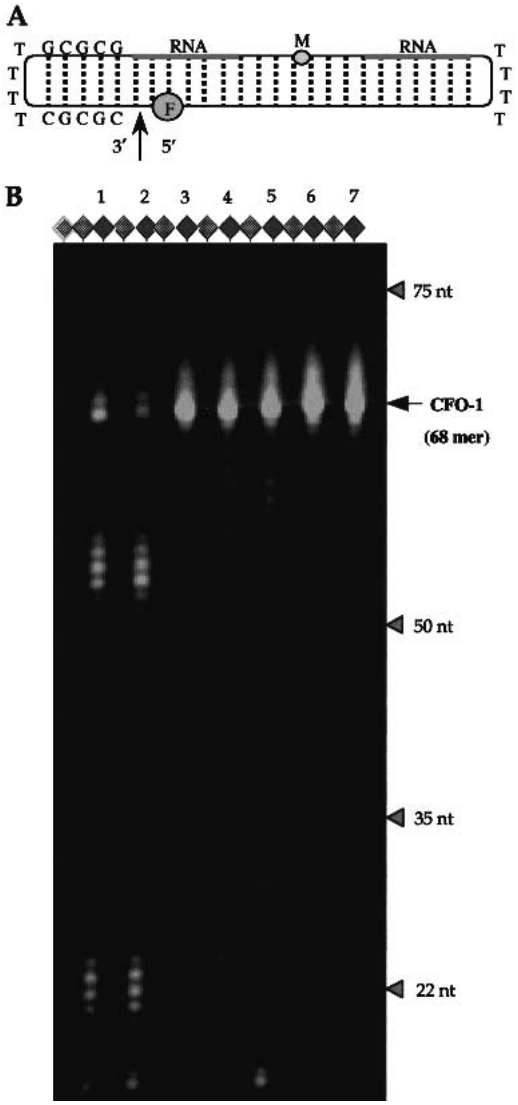


Fig. 1. Schematic representation of a chimeraplast and visualization of CFO-1 chimeraplast degradation patterns. (A) Chimeraplasts are hybrid RNA/DNA oligonucleotides designed for the *in situ* correction of genomic point mutations by specific homologous pairing and mismatch repair stimulation. Two complementary domains of 30 nucleotides, flanked by two T-hairpin loops, which provide stability and prevent concatemerization, compose the original structure of a typical chimeraplast. One of the 30-mer domains carries two 10-mer segments with a 2′-*O*-methyl RNA backbone flanking the mutator region (5 nucleotides). This intermediate DNA region is completely homologous to the genomic sequence holding the mutation except for the mismatched nucleotide (M). RNA-DNA interactions increase the half-life of the chimeraplast/genomic DNA complex within the cells. Moreover, the 2′ modification of the RNA residues protects against enzymatic degradation. For tracking purposes, the 5′ end of the chimeraplast has been modified to include a fluorescent FITC molecule (F). The arrow indicates the strand break allowing interwinding of the oligonucleotide and its target. (B) Digitized gel image of the CFO-1 chimeraplast fluorescent degradation patterns. Lanes 1 and 2, CFO-1 incubated for 24 h and 48 h, respectively, in medium containing 5% fresh FCS; lanes 3 and 4, CFO-1 incubated for 24 h and 48 h, respectively, in medium containing 5% old FCS; lane 5, CFO-1 incubated for 24 h in medium containing 5% inactivated FCS; lane 6, CFO-1 incubated for 24 h in medium without FCS; lane 7, CFO-1 oligonucleotide control. TAMRA-labeled GeneScan-500 size standards are marked on the right. The arrow indicates the position of the 68-mer full-length CFO-1 chimeraplast.

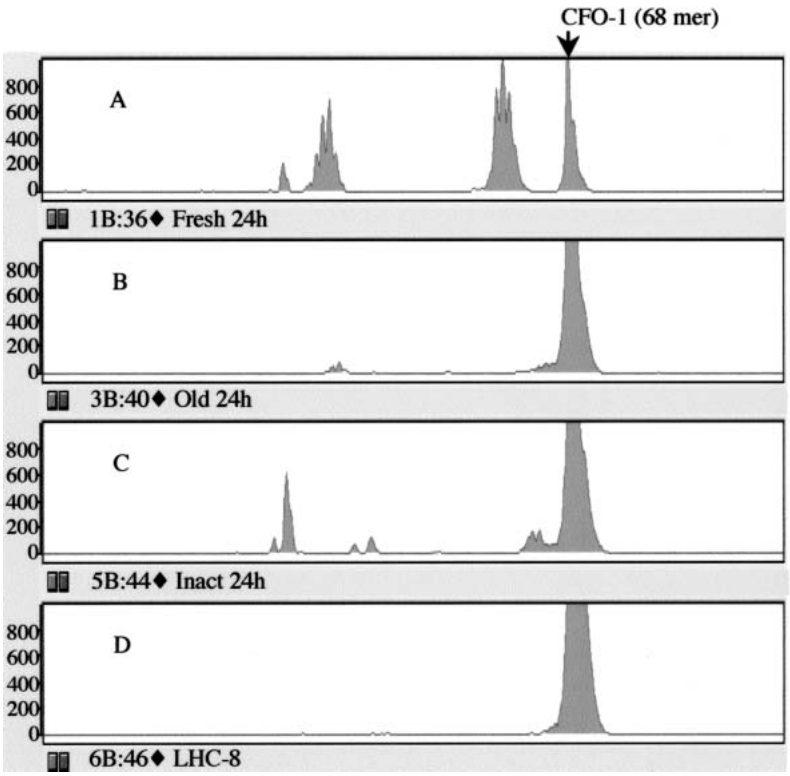
long-term storage of fetal calf serum seemed to abrogate exo- and endonuclease activities to a great extent (Fig. 2B and Table I). Santana *et al.* (13) studied intracellular chimeraplast stability in different epithelial cell lines and showed about the same amount of intact and degraded ^{32}P end-labeled RNA/DNA oligonucleotide at 24–48 h posttransfection. However, a true quantitative analysis of chimeraplast metabolism was lacking in their analysis. This was likely due to the poor resolution of the radiolabeled gel obtained, which also made impossible the assessment of the corresponding nuclease degradation patterns.

Our results indicate that the CFO-1 chimeraplast is unstable in contact with serum, contrarily to what has been reported (10). Thus, the possibility of significant chimeraplast metabolism should be taken into account to assess the efficiency and efficacy of these chimeric RNA/DNA molecules in targeted gene correction assays, either *in vitro* or *in vivo*. The source and condition of the serum component in the culture medium that Yoon *et al.* (10) used in the *in vitro* experiments clearly could have influenced the degree of metabolism of the chimeraplasts, as we have shown by incubation of CFO-1 either with fresh, old, or complement-inactivated serum (Table I). Alternatively, considering the model structure of chimeraplasts as a double hairpin A-form helix (14), the close proximity of their 5′- and 3′-ends and the rather bulky fluorescein label attached to the 5′-end of CFO-1 could have destabilized the chimeraplast, making its 3′-end more accessible to exonuclease degradation. Nevertheless, the CFO-1 oligonucleotide proved to be useful in analyzing different cell culture conditions for susceptibility to chimeraplast degradation.

Additionally, we noted that complexing the chimeraplasts either with PEI or with the cationic lipid cytofectin, which have been used previously in chimeraplast-mediated correction experiments (7), resulted in partial CFO-1 protection against serum nucleases only when presented in the form of PEI polyplexes (Table I). The CFO-1 degradation pattern obtained when complexed with the nonviral vectors was similar to that obtained if incubated naked (data not shown). It is well-known that nonviral carrier systems can confer some degree of nucleic acid protection against nuclease degradation. Biophysical and biochemical parameters influencing cytofectin/chimeraplast and PEI/chimeraplast complex assembly, compactation, aggregation, size, and charge distribution are likely playing an important role in chimeraplast stability toward biological fluids. Indeed, a recent study suggested that PEI but not cationic lipid improved both stability and antisense activity of 3′-capped phosphodiester oligonucleotides (15).

Thus, avoiding as much as possible the nuclease activity present in the serum (e.g., by complement inactivation) or using serum-free media should be desirable in *in vitro* experiments aimed to test uptake and *in situ* mutation repair efficacy of different chimeraplast structures. Further chimeraplast protection, especially for *in vivo* assays, could be conferred by improved galenics, either using novel nonviral vectors with full protection capabilities or by modifying the oligonucleotide chemistry.

All together, these results confirm the validity of our approach for the study of oligonucleotide stability in biological fluids. Additional features make this method more suitable than conventional radioactivity-labeling protocols to assess oligo-



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area
1B, 30	26.88		211	1986
1B, 31	27.95		69	656
1B, 32	28.21		287	1979
1B, 33	28.45		584	4234
1B, 34	28.72		703	5788
1B, 35	28.96		293	2588
1B, 41	35.33		793	7067
1B, 42	35.57		1006	8080
1B, 43	35.84		761	9897
1B, 45	38.16		1166	9680
1B, 46	38.37		542	5514
3B, 18	28.83		56	471
3B, 19	29.07		89	694
3B, 29	36.99		56	515
3B, 30	37.28		76	771
3B, 31	37.52		74	488
3B, 32	37.60		77	374
3B, 33	38.24		4607	62609
5B, 29	26.51		128	920
5B, 30	26.99		625	6401
5B, 33	29.71		73	699
5B, 34	30.35		126	1350
5B, 40	36.75		170	2463
5B, 41	37.01		180	2152
5B, 42	37.60		77	780
5B, 43	38.27		4814	70028
6B, 48	37.65		84	1045
6B, 49	38.35		4886	88711

Fig. 2. Quantitative assessment of CFO-1 chimeraplast degradation by GeneScan analysis. GeneScan expanded view of single-lane electrophoretograms for automatic determination of CFO-1 chimeraplast degradation. Results shown in tabular form include elution time and amount of fluorescence signal in peak height and peak area. Internal-lane size standards have been omitted in the electrophoreses to avoid the possibility of cross-talk between dyes, which could interfere in the quantitative analysis. Electrophoretograms displayed correspond to some of the samples run on the Fig. 1 gel: (A) CFO-1 after 24-h incubation in medium containing 5% fresh FCS; (B) CFO-1 after 24-h incubation in medium containing 5% old FCS; (C) CFO-1 after 24-h incubation in medium containing 5% inactivated FCS; (D) CFO-1 after 24-h incubation in medium without FCS. For each of the sample profiles, all peak height values below 50 were considered background and therefore were not included in the relative quantification analyses.

nucleotide degradation. Fluorescence-labeled oligonucleotides are usually more stable than radiolabeled oligonucleotides, which makes the former more economic, safer, and less bothersome for repeated use. Moreover, GeneScan automated fluorescent analysis enables the simultaneous study of up to four different oligonucleotide structures in the same sample without interference by labeling them with four different fluorescent labels, visualized as blue, green, yellow, and red.

In conclusion, our method combines the excellent performance of slab gel electrophoresis, downward to single-nucleotide resolution, with a broad linear dynamic range of fluorescence laser detection and the analytical power of GeneScan software for quantitative assessment of oligonucleotide degradation, without the need for radioactivity and autoradiography. The reliability and simplicity of this approach should be valuable in a variety of pharmacokinetic experiments directed to the development of more efficacious oligonucleotide-based therapeutics.

Table I. CFO-1 Chimereplast Stability in Culture Medium under Different Serum Conditions

	% CFO-1 oligo degradation[a]	
Sample	24 h	48 h
CFO-1 + LHC-8 with 5% fresh FCS	73 (±5)	89 (±3)
CFO-1 + LHC-8 with 5% old FCS	8 (±7)	11 (±10)
CFO-1 + LHC-8 with 5% inactivated FCS	19 (±7)	n.d.
CFO-1 + LHC-8 without FCS	1 (±1)	n.d.
CFO-1/PEI + LHC-8 with 5% fresh FCS	47 (±6)	80 (±9)
CFO-1/cytofectin + LHC-8 with 5% fresh FCS	77 (±6)	89 (±9)

Note: n.d.: not determined; SD: standard deviation.

[a] CFO-1 oligonucleotide degradation was calculated as the sum of all fluorescent peaks of a given electrophoretogram sample excluding the peak from full-length CFO-1, divided by the sum of all fluorescent peaks from the sample including the peak from full-length CFO-1, and given as a percentage mean (±SD) of three different experiments.

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