Ex vivo regulation of specific gene expression by nanomolar concentration of double-stranded dumbbell oligonucleotides

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ABSTRACT

Inhibition of specific transcriptional regulatory proteins is a new approach to control gene expression. Transcriptional activity of DNA-binding proteins can be inhibited by the use of double-stranded (ds) oligodeoxynucleotides that compete for the binding to their specific target sequences in promoters and enhancers. As a model, we used phosphodiester dumbbell oligonucleotides containing a binding site for the liver-enriched transcription factor HNF-1 (Hepatocyte Nuclear Factor 1). Binding affinity of HNF-1 to dumbbell oligonucleotides was the same as that to ds oligonucleotides, as determined by gel retardation assays. HNF-1 dumbbells specifically inhibited *in vitro* transcription driven by the albumin promoter by more than 90%. HNF-1-dependent activation of a CAT reporter plasmid was specifically inhibited when the HNF-1 dumbbell oligonucleotide was added at nM concentration to transiently transfected C33 cells. On the contrary, HNF-1 ds oligonucleotides, which displayed the same activity as the dumbbell oligonucleotides in the *in vitro* assays, were no more effective in the *ex vivo* experiments. These results might reflect the increased stability of the circular dumbbell oligonucleotides towards cellular nuclease degradation, as shown *in vitro* with nucleolytic enzymes. Dumbbell oligonucleotides containing unmodified phosphodiester bonds may efficiently compete for binding of specific transcription factors within cells, then providing a potential therapeutic tool to control disease-causing genes.

INTRODUCTION

Transcriptional control in eukaryotes results from the interplay between *cis*-acting DNA sequences in promoters, enhancers or silencers and *trans*-acting regulatory proteins (1, 2). Transcription initiation involves the formation of a complex between RNA polymerase II and several auxiliary factors at the TATA box (3) or at the initiator element (4), to give the basal level of promoter activity (5–7). The activity of the basal transcriptional machinery

is further modulated by the combined action of sequence-specific DNA binding factors (1, 2) and mediator or coactivator proteins, which may facilitate the formation of protein complexes between the DNA-binding proteins and the RNA polymerase II basal initiation machine (8, 9).

Transcription factors constitute potential targets for regulating gene expression. One possible approach to decrease the availability of a particular DNA-binding protein and, consequently, to decrease or activate the expression of a specific gene, is to titrate the targeted protein with its specific binding site. This 'sense' approach then provides a potential tool to regulate the expression of specific genes involved in viral diseases or cancer. Such competition mechanism has often been observed when studying promoter activity in transient expression assays. Promoter competition can take place between cotransfected plasmids containing *cis*-acting elements in common or even when a single plasmid is introduced in the cells at high concentration (10–12). Transcription factors are also able to bind *in vitro* to synthetic double-stranded oligonucleotides and such property has been used to analyze the regulation of different promoters. For example, double-stranded oligonucleotides containing the binding sites for HNF-1, SP1 or BPV-1 E2 were shown to inhibit *in vitro* RNA transcription driven by these DNA-binding proteins in a concentration-specific manner (13–15).

Double-stranded oligonucleotides were also shown to compete *ex vivo* with cellular promoter sequences for the binding of transcription factors. Double-stranded oligonucleotides carrying the major c-fos CRE site are sufficient to block induction of the endogenous c-fos gene by cAMP when microinjected in 3T3 cells (16). In addition, microinjection of AP-1 double-stranded oligonucleotides blocks DNA synthesis in response to growth factors which normally induce cell cycling in human Hs68 fibroblasts (17). On the other hand, double-stranded phosphorothioate oligodeoxynucleotides containing the binding sites for NF- κ B or Oct-1 were shown to inhibit HIV-CAT or Oct-CAT expression when cotransfected with these reporter vectors in an Epstein–Barr virus-transformed B cell line (18).

We are interested in studying phosphodiester double-stranded oligonucleotides. Unmodified dumbbell oligonucleotides are an attractive model of competitor oligonucleotides, since they

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combine proper binding of transcription factors (19) together with an increased stability towards nucleolytic degradation due to the absence of free termini. We have analyzed the ability of dumbbell oligonucleotides to specifically regulate gene expression. As a model system, we have studied the activity of a promoter controlled by HNF-1 (Hepatocyte Nuclear Factor 1). HNF-1 is a transcription factor involved in the regulation of a wide variety of liver-specific genes (reviewed in 20). For example, the binding of HNF-1 is strictly necessary for the expression of the mammalian albumin promoter (13, 21–23). Here, we show that dumbbell oligonucleotides containing an HNF-1 binding site are able to specifically inhibit the albumin promoter activity both *in vitro* and *ex vivo* at nM concentrations.

MATERIALS AND METHODS

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems model 394/8 DNA synthesizer, using phosphoramidite chemistry. 5' phosphate oligonucleotides were synthesized using 5' Phosphate-On cyanoethyl phosphoramidite (Clontech Lab., Inc.) as the phosphorylating reagent.

Sequences of the oligodeoxynucleotides used in this study are depicted in Table I. PE56 double-stranded oligonucleotide (dsPE56) contains the rat albumin sequence –63/–41 which encompasses the HNF1 binding site (13). DS34 contains four mutated bases which abolish completely HNF1 binding (13). GT-56_o and GT-56_c, and GT-34_o and GT-34_c are the corresponding non-ligated and ligated PE56 and DS34 dumbbell oligonucleotides.

Synthesis of ligated dumbbell oligonucleotides

Ligated oligodeoxynucleotide dumbbells GT-56_c or GT-34_c (Table I) were obtained by ligation of the corresponding 5' phosphate oligonucleotides GT-56_o or GT-34_o with T4 DNA ligase. Standard ligation conditions were: 15 μ M oligonucleotide, 50 mM Tris HCl pH 7.8, 10 mM MgCl₂, 20 mM DTT, 1 mM ATP, 1 mM BSA and 10.000 units/ml of T4 DNA ligase incubated in a total volume of 1 ml at 4°C for 48 hours. After ligation, reaction mixtures were concentrated by ethanol precipitation and ligated products were separated on a 12% polyacrylamide gel containing 7M urea. In each case, UV shadowing revealed a minor band comigrating with the GT-56_o or GT-34_o non-ligated oligonucleotide, along with a major new product which migrated more slowly than the respective non-ligated oligonucleotide. With other particular sequences, electrophoretic migration of the major ligation product may be similar, slower or faster than the corresponding non-ligated oligonucleotide. The major ligation product was excised and eluted from the gel, precipitated with ethanol and quantitated by absorbance at 260 nm. The identity of the ligated dumbbells was confirmed by comigration with radioactive standards (see below).

Radioactive ligated dumbbells were obtained as follows. 5' phosphate GT-56_o or GT-34_o oligonucleotides were first incubated with calf intestine phosphatase under standard conditions and the dephosphorylated oligonucleotides were recovered by ethanol precipitation after phenol extraction. Two pmoles of oligonucleotide were phosphorylated with 30 μ Ci (γ^{32} P)-ATP (3000 Ci/mmol) and T4 polynucleotide kinase, and then ligated with T4 DNA ligase and purified by denaturing polyacrylamide gel electrophoresis, as described for non-

Table I. Oligonucleotides

dsPE56	⁻⁶³ TGTGGTTAATGATCTACAGTTA CACCAATTACTAGATGTCAATA ⁻⁴¹
GT 56 _o	T T T T T T TGTGGTTAATGATCTACAGTTAT A CACCAATTACTAGATGTCAATA T T T T T T
GT 56 _c	T T T T T T TGTGGTTAATGATCTACAGTTAT ACACCAATTACTAGATGTCAATA T T T T T T
GT 34 _c	T T T T T T TGTGGTgATGAggTACAGTTAT ACACCAcATACTcCATGTCAATA T T T T T T

Table I depicts the structure and the sequence of the oligonucleotides used in this study, as described in the text. dsPE56 contains the rat albumin promoter sequence –63/–41 which includes the HNF-1 binding site (13). GT-56_o corresponds to the open HNF-1 dumbbell structure, the position of the nick being indicated by 5'P. GT-56_c corresponds to the closed HNF-1 dumbbell structure, obtained by ligation of GT-56_o as described in Mat.&Meth. The 15 bp pseudopalindromic binding site of HNF-1 is underlined in each oligonucleotide. GT-34_c corresponds to a closed dumbbell oligonucleotide containing four mutated bases in the HNF-1 binding site (indicated by lower case letters) which completely abolish HNF-1 binding (13).

radioactive dumbbells, except that radioactive bands were detected by autoradiography and quantified by liquid scintillation counting. The identity of the ligated dumbbells was verified by incubation with alkaline phosphatase, phosphodiesterase I or by S1 nuclease protection mapping.

Enzymatic characterization of ligated dumbbell oligonucleotides

Treatment with phosphodiesterase. Reaction mixtures containing 10 μ M non-ligated or ligated radioactive oligonucleotide (10⁵ cpm/ μ g), 50 mM Tris HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT and 5 $\times$ 10⁻⁵ units of phosphodiesterase I in a final volume of 10 μ l, were incubated at 37°C for 30 min. After incubation, the reaction was stopped by heating to 75°C for 10 min; then, 10 μ l of formamide loading buffer were added and reaction products were analyzed on a 15% polyacrylamide gel containing 7 M urea. Radioactive bands were detected by autoradiography, and labeled products were quantified by excision from the gel and liquid scintillation counting.

Treatment with nuclease S1. Reaction mixtures contained 10 μ M non-ligated or ligated radioactive oligonucleotide (10⁵ cpm/mg), 50 mM NaCl, 33 mM sodium acetate pH 4.4, 30 μ M ZnSO₄ and 1 unit of S1 nuclease in a final volume of 25 μ l. After incubation at 37°C for 20 min, the reaction was stopped by adding 20 mM EDTA, 0.3 M sodium acetate and 20 μ g glycogen; oligonucleotides were then precipitated with ethanol. After washing and drying, the DNA pellets were resuspended in 10 μ l of formamide loading buffer and reaction products were analyzed as described above.

Preparation of liver nuclear extracts

Nuclear extracts from rat liver were prepared as described in ref.13. Protein concentration was determined by the BCA protein assay reagent (Pierce).

Gel retardation assays

Binding reactions for band shift assays were performed in 14 μ l of reaction mix containing: 10 mM Hepes pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% (vol/vol) glycerol, 0.25 mM PMSF, 2.5 μ g/ml aprotinin, 2.5 μ g/ml leupeptin, 6 mM $MgCl_2$, 6 mM spermidine, 1.5 μ g poly (dI-dC)·poly (dI-dC), 250 ng sonicated salmon sperm DNA, 3 fmol of (32 P)-labeled oligonucleotide probe and 0.2–5 μ g of liver nuclear protein. Where indicated, specific or non-specific oligonucleotide competitors were added at the same time as the radioactive probe. After incubation at 4°C for 10 min, reaction mixtures were loaded onto a 6% polyacrylamide non-denaturing gel containing 0.25×TBE and were electrophoresed at 12 V/cm until a suitable separation was achieved. The gel was fixed, dried and exposed to X-ray film. Radioactive bands were quantitated by liquid scintillation counting.

Plasmid constructions

pALB-GF contains the rat albumin promoter cloned in front of a 375 bp G-free cassette. The albumin sequence was obtained by ligation of six overlapping double-stranded oligonucleotides as described in ref.24, except that the TF6/TF7 oligonucleotides were replaced by

5' GTTAGAGAAGTATATTAGAGCGAGTG 3'
3' TCTCTTCATATAATCTCGCTCACTTAA 5'

thus generating a –152/–14 rat albumin sequence flanked by BglII and EcoRI 5' and 3' cohesive ends, respectively. This promoter was cloned between the BglII and EcoRI sites from p(C₂AT)₁₉BE, obtained by inserting a polylinker containing unique BglII, XbaI, XhoI and BamHI restriction sites in the EcoRI site from p(C₂AT)₁₉ (25). Since the inserted promoter lacks the transcription start site, initiation takes place in the vector sequences giving a transcript of about 370 nt. pML(C₂AT)₁₉ (25) contains the adenovirus 2 major late promoter (–400/+10) linked to a 375 bp G-free cassette, generating a 385 nt transcript. In pAlb400 (26), the mouse albumin promoter (–650/+22) directs the transcription of a 398 nt G-free transcript.

pΔE1 Alb-CAT (27) contains CAT sequences under the control of the rat albumin promoter (–151/+16). pCH110 codes for *E. coli* β -galactosidase under the control of the SV40 early promoter (28). pRSV-HNF1 contains the cDNA for the rat transcription factor HNF1 under the control of the RSV LTR (29)

In vitro transcription assays

Transcription assays were performed using the 'G-free cassette' system developed by Sawadogo and Roeder (25). Reactions were carried out in a volume of 20 μ l of 25 mM Hepes pH 7.6, 50 mM KCl, 10% glycerol, 6 mM $MgCl_2$, 0.6 mM ATP and CTP, 0.1 mM 3-O-methyl-GTP, 7 μ Ci (α - 32 P)-UTP (3000 Ci/mmol), 5 μ M UTP, 6 nM of pAlb400 or pAlbGF specific template DNA, 2 nM of pML(C₂AT)₁₉ control DNA, 30 units of RNasin. and 1.6 mg/ml of liver nuclear protein. Transcription efficiency was optimized by varying the relative amounts of DNA template and nuclear extract. DNA templates were preincubated with extracts for 10 min on ice, and transcriptions were initiated by adding the nucleotides. After incubation at 30°C for 1 h, the reactions were stopped by adding 280 μ l of 20 mM Tris pH 7.5, 0.25 M NaCl, 1% SDS, 5 mM EDTA, followed by digestion with 40 μ g of proteinase K for 30 min at 37°C. The transcription products were then extracted with phenol, precipitated with ethanol and

Figure 1. Stability of dumbbell oligonucleotides. This figure shows the autoradiograph of the products obtained by incubation of GT-56_c (right panel) or GT-56_o (left panel) with phosphodiesterase I or S1 nuclease, as indicated. Enzymatic reactions were performed as described in Mat. & Meth. Lane M contains synthetic oligonucleotides as size markers, 21, 22 and 26 nucleotides (nt) as indicated by arrows. The markers correspond to the upper strand of dsPE56 (22-mer, Table I), or to the upper strand of dsPE56 (Table I) without the 5' terminal T (21-mer), or to the upper strand of dsPE56 (Table I) plus two T on each 5' and 3' ends (26-mer). The right and left schemes depict the size of the expected radioactive products obtained by total (NO LOOP) or partial (PARTIAL LOOP) and WHOLE LOOP degradation by S1 nuclease of the single stranded regions in GT-56_c or GT-56_o, respectively. CONTROL, untreated oligonucleotides; PDEI, snake venom phosphodiesterase I; S1, S1 nuclease.

analyzed by electrophoresis on a 4% acrylamide/7 M urea gel. Gels were dried and exposed to X-ray films at –80°C with intensifying screen.

DNA transfection and transient CAT assay

C33 human epithelial tumour cells (30) were cultured in DMEM supplemented with 10% fetal calf serum and were transfected by the standard calcium phosphate coprecipitation protocol (31). Falcon dishes (6 cm) containing 5×10^5 C33 cells, were transfected in triplicates with a mixture of pΔE1 Alb-CAT (2 μ g) and pRSV-HNF1 (0.5 μ g), plus 0.5 μ g of pCH110 as control for transfection efficiency; the total amount of DNA transfected was maintained constant (5 μ g) by addition of a RSV-luciferase plasmid. Where indicated, oligonucleotides were included in the calcium phosphate-DNA precipitate. The precipitate was left on the cells overnight, and cell extracts were prepared 48 hours after transfection. CAT activity was measured by the phase-extraction assay and liquid scintillation counting, using 14 C-chloramphenicol and butyryl CoA as substrates (31). β -galactosidase activity was determined by standard procedures (31). Values lower than twice the background obtained in non transfected cells were discarded.

RESULTS

Enzymatic characterization of circular dumbbell oligonucleotides

In many eukaryotic systems, antisense or sense oligonucleotides have been used to inhibit gene expression specifically. The

Figure 2. Band shift assays and competitions with HNF1 dumbbells. **A** shows the autoradiograph of a gel retardation assay using increasing amounts of liver nuclear extracts and HNF1 probes in the form of double-stranded oligonucleotide (dsPE56), open dumbbell (GT-56_o) or closed dumbbell (GT-56_c), as indicated. In **B**, the binding assay was performed using 1 µg of liver nuclear extract (+) and 3 fmol of 5' end-labeled GT-56_o probe, in the absence (–) or the presence (+) of a 50-fold molar excess of unlabeled single stranded PE56 upper oligonucleotide (ss COMPETITOR; see Table I). **C**: Autoradiograph of a gel retardation assay carried out with 1 µg of liver nuclear extract and 3 fmol of ³²P dsPE56, in the presence of the indicated amounts of unlabeled dsPE56, GT-56_c or GT-34_c oligonucleotides. Lanes designed 0 contained no competing oligonucleotides. The arrows indicate the positions of the HNF1 specific complex and the free probes, respectively.

application of oligonucleotides *in vivo* however, may be severely hampered by their sensitivity to nucleases that makes them unstable in a biological environment. It has recently been reported that degradation of unmodified phosphodiester oligodeoxynucleotides in cultured cells, culture media and serum, takes place primarily as a result of 3' exonuclease activity (32–34). The serum 3' exonuclease shows a strong preference, if not an absolute requirement, for single-stranded substrates and has no associated 5' exonuclease or endonuclease activities, but may have a phosphatase associated activity (33–35; E.U., N.E. and M. B., unpublished results).

To overcome the problem of losing the 5' label in the non-ligated dumbbell by dephosphorylation in serum, we compared the stability of the open or closed dumbbells to 3' exonucleolytic degradation by incubating them with phosphodiesterase I, a well-characterized 3' exonuclease from snake venom. As shown in Figure 1, as expected for a closed molecule, the ligated dumbbell GT-56_c was mostly resistant to degradation (right panel), while under the same conditions the open dumbbell precursor GT-56_o was almost completely degraded by PDEI (left panel).

We further analyzed the structure of GT-56_o and GT-56_c by incubating them with S1 nuclease. This enzyme will attack the single-stranded regions of the dumbbells (the interstrand connecting loops), thus allowing to recover the double-stranded portion of the oligonucleotide. In the case of the non-ligated dumbbell GT-56_o, the radioactive label should be associated to a 5' end-labeled 12-mer, while for the ligated dumbbell GT-56_c the radioactive strand should be a 23-mer inner-labeled oligonucleotide (see Table I).

As shown in Figure 1, protected radioactive fragments of 20 to 30 nucleotides were obtained for GT-56_c (right panel), clearly indicating that GT-56_c corresponds to a ligated dumbbell in which the complementary sequences are mostly in a double-

stranded structure. Moreover, the fact that the predominant protected bands were longer than 23 nt (up to 27 bases, Fig. 1, right panel) might suggest that the dumbbell structure can interfere with the endonucleolytic degradation of the loops. On the contrary, in the case of the non-ligated precursor GT-56_o, no 12-mer radioactive fragment was observed (Fig. 1, left panel). This result indicates that the (³²P) label present in the nick of GT-56_o is accessible to degradation by S1 nuclease, and probably suggests that the complementary region in the non-ligated dumbbell is at least partially deshybridized.

Similar results were obtained upon analysis of GT-34_o and GT-34_c (data not shown).

Specific binding of the liver transcription factor HNF-1 to a closed dumbbell carrying the HNF-1 target site

We have next investigated the ability of a targeted DNA-binding protein to discriminate between specific and non-specific dumbbells in an *in vitro* binding system. For this purpose we have chosen HNF-1 (Hepatocyte Nuclear Factor 1), a well characterized liver-enriched transcription factor which binds as a dimer to a 15 bp pseudopalindromic site present in the promoters or enhancers of several hepatic-specific genes (29, 36).

We have first compared the relative binding affinity of rat liver HNF-1 towards an HNF-1 site contained in a double stranded oligonucleotide, in an open dumbbell oligonucleotide or in a closed dumbbell oligonucleotide (Table I, dsPE56, GT-56_o or GT-56_c, respectively). This particular HNF-1 binding site corresponds to the sequence present in the rat albumin promoter, which has been shown to be a high affinity site when compared to other natural HNF-1 targets or to mutated sites (13). As shown in Figure 2-A, when we incubated radioactive dsPE56 or GT-56_c probes with increasing amounts of liver nuclear proteins, a DNA–protein complex corresponding to the HNF-1

retarded band was readily observed. On the contrary, two DNA-protein complexes were detected with the non-ligated dumbbell probe GT-56_o: one migrating at the HNF-1 position and another band migrating faster. The latter band might correspond to a single-stranded DNA-binding protein, since it was competed with a 50-fold molar excess of unlabeled single-stranded oligonucleotide (Fig. 2B).

Quantitative analysis, taking into account the specific activity of each probe, revealed that HNF-1 binds to a closed dumbbell carrying its specific recognition sequence with the same relative affinity as to a double-stranded target site. In contrast, a five-fold decrease of HNF-1 retarded band was observed with the non-ligated dumbbell probe, GT-56_o, together with the formation of a different complex that can be competed by a single-stranded oligonucleotide. Taken together, these data may indicate that the stability of the double-stranded structure in the non-ligated dumbbell is lower than that of the ligated oligonucleotide, probably being displaced to a partial single-stranded form.

Similar results were obtained by comparing the relative binding efficiencies of the different oligonucleotides in competition experiments. Again, as for the direct gel retardation assay using each oligonucleotide as a probe, addition of unlabeled GT-56_c or ds PE56 competed for HNF-1 binding to radioactive ds PE56 in the same way (Fig. 2C), while unlabeled GT-56_o was about five-fold less effective competitor (data not shown).

Finally, in order to assess the specificity of the binding to a dumbbell oligonucleotide, we synthesized a closed dumbbell that contains an HNF-1 mutated site, GT-34_c (Table I), that totally abolishes HNF-1 binding (13). The mutated closed dumbbell was then analyzed in a competition experiment. Addition of a 15-fold molar excess (Figure 2C) or even a 600-fold excess (not shown) of cold GT-34_c did not compete with labeled PE56 for HNF-1 binding; under the same conditions, GT-56_c or ds PE56 almost completely inhibited the binding of the radioactive probe (Fig. 2C).

Essentially similar results were obtained by analyzing the behaviour of dumbbells containing the binding sequence for the CCAAT binding factor NF-Y or Sp1 (data not shown)

Dumbbell oligonucleotides can specifically inhibit RNA synthesis in an *in vitro* transcription assay

HNF-1 is a homeodomain-containing transcriptional regulator that is essential for the liver-specific expression of albumin and many other hepatic genes, including seroproteins, enzymes involved in carbohydrate metabolism or detoxification (reviewed in 20). *In vitro* studies confirmed the crucial role of HNF-1 in hepatocyte-specific transcription: mutation of the HNF-1 binding site drastically reduces the transcription of the albumin promoter (22; M.B., unpublished results) and, in addition, purified HNF-1 is sufficient to complement a spleen nuclear extract for the *in vitro* transcription of the mouse albumin promoter (23). The *in vitro* transcription of the albumin promoter is then a suitable model for studying the effect of HNF-1 dumbbells as specific inhibitors of transcription.

We used two different albumin-specific plasmids: pAlb400, which contains the mouse albumin promoter and pALB-GF, which contains the rat albumin promoter. The pAlb400 mouse albumin construct contains the promoter sequences from position -650 to +22 and gives a G-free transcript of about 400 nucleotides; an equivalent construction from the rat albumin promoter cannot be used in this assay because there is a G-residue

Figure 3. Competition of HNF1 dumbbells in an *in vitro* transcription assay. The autoradiograph shows a transcription assay using liver nuclear extracts with 2 nM of pML(C₂AT)₁₉ internal control template (adenovirus major late promoter G-free cassette) and 6 nM of pALB-GF (rat albumin G-free cassette) or 6 nM of pAlb400 (mouse albumin G-free cassette), in the presence of 60 nM dsPE56, GT-56_c, GT-56_o or GT-34_c dumbbells, as indicated. Lanes designed CONTROL contained no competing oligonucleotides. AdML indicates the adenovirus major late promoter transcript (385 nt); mAlb indicates the 400 nt transcript from the mouse albumin promoter; rAlb indicates the 370 nt transcript originated from the rat albumin promoter.

at the +14 position. On the other hand, transient expression assays indicated that the first 150 bp of the rat albumin 5'-flanking region are necessary and sufficient for tissue- or cell-specificity (27). Therefore, the rat sequences used for the *in vitro* transcription studies (pALB-GF) corresponded to the fragment -152 to -14 from the albumin promoter, and showed weaker activity than the -650/+22 mouse promoter. Since the rat promoter inserted in pALB-GF lacks the transcription start site, initiation takes place in the vector sequences giving a transcript of about 370 nt.

As shown in Figure 3, when liver nuclear extracts were used to transcribe 6 nM of pALB-GF in the presence of 2 nM of pML(C₂AT)₁₉, the albumin-specific transcript was transcribed at about the same level as the adenovirus major late promoter used as a control. On the contrary, when transcription was carried out in the presence of 60 nM of dsPE56 or GT-56_c, the albumin-specific transcription was reduced to 5–10%, while the adenovirus control was not affected. It is interesting to note that transcription of a template containing the DS34 mutation in the HNF-1 site (Table I) also gives 10% of the wild type template transcription (M.B., unpublished results), thus indicating that GT-56_c is able to quantitatively compete for all the HNF1-dependent transcription. Most important, the specificity of the HNF1-dumbbell interaction was not modified in the *in vitro* transcription system, since a 10-fold (Fig. 3) or a 50-fold molar excess (not shown) of GT-34_c HNF-1 mutant dumbbell did not affect the albumin-specific signal.

Finally, addition of GT-56_o was about as effective as dsPE56 or GT-56_c (Fig. 3). One possible explanation for the difference between the *in vitro* transcription (Fig. 3) and the binding results (Fig. 2) is that only a fraction of the albumin G-free plasmids is active for transcription; then, the molar ratio of competitor oligonucleotide to promoter sites would be greater than 10 and would not allow to distinguish a 5-fold difference in binding affinity, as detected in the gel retardation experiments.

Similar results were obtained when pAlb400 was used (Figure 3, right panel). As indicated above, this mouse promoter construction is transcribed about 10-fold more than the ALB-GF rat promoter. As shown in Fig. 3 (right panel), addition of either oligonucleotide resulted in about 90% inhibition of the

Figure 4. Inhibition of HNF1 transcriptional activity in C33 cells by HNF1 dumbbells. GT-56_c, GT-56_o, ds PE-56 or GT-34_c oligonucleotides were cotransfected in C33 cells with pΔE1 Alb-CAT reporter vector and pRSV-HNF1 expression vector at the indicated concentrations, as described in Mat. & Meth. Transfections were performed in triplicates; transfection efficiency was determined using pCH110 (SVe-β gal) as internal control. 100% refers to the maximal CAT activity obtained at 2nM GT-56_o, GT-34_c or ds PE56.

control transcription. As explained before, this is the level of transcription obtained with a promoter mutated at the HNF-1 site, and therefore, it represents most probably HNF-1 independent transcription. Since GT-56_c was able to almost completely inhibit the HNF-1-dependent transcription, we concluded that the dumbbell competition was not restricted to a particular promoter construction and could even occur with a very active promoter.

Dumbbell oligonucleotides can specifically inhibit CAT activity in a transient transfection assay

The fact that HNF-1 dumbbells were shown to correctly bind to HNF-1 in *in vitro* assays prompted us to test the ability of the dumbbell oligonucleotides to regulate gene expression in an *ex vivo* cellular system. C33 human epithelial tumour cells do not express the HNF-1 protein, and therefore, cannot support the expression of a transfected CAT vector controlled by the HNF-1 dependent albumin promoter (pΔE1 Alb-CAT). Cotransfection with an HNF-1 expression vector (pRSV-HNF-1) allows expression of the CAT protein proportionally to the quantity of the HNF-1 expression vector added (37).

In order to evaluate the competition efficiency of the dumbbell oligonucleotides, we set up the conditions for cotransfecting C33 cells with pΔE1 Alb-CAT and subsaturating amounts of pRSV-HNF1. We then cotransfected pΔE1 Alb-CAT and pRSV-HNF1 with GT-56_o, GT-56_c, GT-34_c or ds PE56 oligonucleotides at a molar ratio of pΔE1 Alb-CAT to oligonucleotide equal to 1:1 or 1:5.

As shown in Figure 4, when GT-56_c was added at 2 nM (corresponding to an equimolar addition respect to the HNF-1 sites in the promoter), it inhibited almost 80% of the HNF-1 specific transcription. On the contrary, under the same conditions GT-56_o, ds PE56, as well as the mutant dumbbell GT-34_c, showed maximal promoter activity. At higher concentrations (Fig.4, 10 nM; pΔE1 Alb-CAT:oligonucleotide ratio=1:5), inhibition by GT-56_c was still substantial, while GT-56_o or ds PE56 showed about 30% inhibition. However, 15% inhibition was also observed with the mutant dumbbell GT-34_c, which should normally not interfere with HNF-1 binding. This results indicate that some non-specific effects might take place at 10 nM.

DISCUSSION

Synthetic oligonucleotides provide a potential therapeutic tool to control the expression of specific genes involved in viral diseases or cancer. One of the major problems that has to be solved to increase the *in vivo* biological efficiency of oligonucleotides, is to reduce their sensitivity to nucleases. This is particularly important in the case of antisense single-stranded phosphodiester oligonucleotides, since degradation in cultured cells, culture media and serum, occurs from the 3' end by means of a 3' processive exonuclease (32–34). On the contrary, the use of double-stranded oligonucleotides as competitors for transcription factors in a sense approach, presents the advantage that double-stranded sequences are more stable in serum and intracellularly than single-stranded oligonucleotides (33, 35; E.U., N.E. and M.B., unpublished results). In the present studies, we demonstrated that stabilization of a double-stranded (ds) phosphodiester sequence by connecting both ends with nucleotidic loops (dumbbell structure), allows to detect specific biological activity of sense oligonucleotides at nanomolar concentration, while under the same conditions the ds oligonucleotides are inactive.

The degradation studies here presented suggest that the GT56c dumbbells preferentially adopt a double-stranded structure along the whole complementary stem region, while the 11 bp stem in GT56o appears not to be stable under the reaction conditions. Moreover, the double-stranded closed structure seems to protect the dumbbell oligonucleotides not only from 3' exonucleases but also from endonucleolytic loop degradation. Even in the case of a minor degradation by a cellular single-stranded endonuclease, this would result in the generation of a still functional double-stranded oligonucleotide, thus allowing to consider the closed dumbbell oligonucleotides described here as potential prodrug compounds.

Stabilization of duplexes in hairpin or dumbbell structures has been demonstrated both by thermal denaturation analysis (38) and sensitivity to degradation by nuclear extracts (35). For example, Ma and col. have recently reported that the *T_m* of linker-derivatized TAR RNA duplexes were 24–31°C higher than that of the unlinked duplex (38). However, there was no correlation between *T_m* and protein binding, as measured in a gel shift assay. Also, it has been described that introduction of a nucleotide loop in a duplex may significantly alter the proximal duplex structure (39). Therefore, since sense oligonucleotides perform their biological activity through protein binding, some oligonucleotide flexibility might be required. Thus, it was very important to verify that the targeted proteins were able to correctly bind to dumbbell oligonucleotides. The results presented here concerning the relative binding affinities of the HNF-1 dumbbell as well as that observed for the CAAT binding protein NF-Y or for Sp1 (data not shown), indicate that the presence of interstrand connecting loops in these particular phosphodiester dumbbell oligonucleotides does not modify the protein/oligonucleotide interactions by changing the affinity or the specificity of the binding.

Chu and Orgel have recently reported that dumbbells containing CREB or JUN binding sites can replace the corresponding double-stranded sequences in gel shift assays with equal or greater sensitivity (19). Even though the specificity of the dumbbell recognition was not assessed by the authors since they performed the binding assays with purified CREB or JUN proteins and not with crude nuclear or total cell extracts, these data support the

idea that the use of unmodified dumbbells can be extended to any DNA-binding protein for which the recognition sequence is available. However, care has to be taken concerning the specificity of the protein interaction with some modified dumbbells. For example, by using Pt^{II} complexes to crosslink CREB or JUN to phosphorothioate-containing dumbbells, Chu and Orgel reported that in PC12 nuclear extracts JUN, and not CREB, was crosslinked to the CRE dumbbell (40).

Double-stranded unmodified oligonucleotides containing binding sequences for HNF1 (13), Sp1 (14) or BPV-1 E2 transactivator (15) have been shown to effectively inhibit transcription in eukaryotic *in vitro* systems. Our results indicate that dumbbell oligonucleotides, which are more stable than the double-stranded ones (35), can also be used as specific competitors for the normal assembly of the transcriptional machinery, thus resulting in the inhibition or activation of a particular gene.

It is interesting to note that ds PE56 or GT-56_c were unable to inhibit in the transient expression system, while in the *in vitro* transcription assay they were as effective as GT-56_c (Fig.3). Since oligonucleotides were introduced into cells by cotransfection with the plasmid vectors, their different activity seems not to be related to a different uptake, which has not been addressed by these experiments. On the contrary, these results might reflect the more stable conformation of GT-56_c in the *in vivo* experimental conditions.

Gilman and coworkers reported that microinjected unmodified double-stranded c-fos CRE or AP1 binding sites are able to block c-fos induction by extracellular stimuli or to block initiation of DNA synthesis, respectively, probably by competing for CREB or JUN binding (16, 17). Since the authors used a microinjection procedure, it is difficult to evaluate the concentration effects of the oligonucleotides and their relative stability in the culture medium. Using a transfection approach similar to that described in this section, Bielinska et al. (18) reported efficient inhibition by octamer or NF- κ B unmodified or phosphorothioate double-stranded oligonucleotides at the μ M range of concentration; however, non-specific inhibition at this concentration was about 30%. Our results show that the use of nanomolar concentrations of non-modified dumbbell oligonucleotides results in highly efficient specific inhibition of gene expression.

Taken together, the results presented here suggest that dumbbell oligonucleotides can be used at very low concentrations to specifically regulate cellular gene expression in cells. Dumbbell oligonucleotides are very stable to exonucleolytic degradation, and even if they can be attacked by single-stranded endonucleases, the binding site still remains functional. The limited toxicity of unmodified phosphodiester double-stranded oligonucleotides and the high specificity of target regulation indicate that dumbbell oligonucleotides may be useful in the inhibition of transcription of disease-causing agents.

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