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Title: “Monitoring Gene Expression In Vivo with Nucleic Acid Molecular Switches.”

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Final Progress Report

Description of Funded Research

The overall objectives of this project were 1) to develop allosteric ribozymes capable of acting as molecular switches for monitoring the levels of both wild-type and mutant mRNA species in living cells and whole animals and 2) to develop highly efficient reagents to deliver nucleic acid molecular switches into living cells, tissues and animals with the ultimate goal of expression profiling specific mRNAs of diagnostic or prognostic value within tumors in animals.

During the past year, we have moved our laboratory to Nevada and in the moving process we have lost electronic and paper copies of prior progress reports concerning the construction and biological properties of the molecular switches. Since there was minimal progress during the last year on molecular switches, we are relying on past project reports to provide a summary of our data on this facet of the grant. Here we are summarizing the work done on the delivery reagents and their application to inducing mutations in living cells, which will include work done during the no cost extension. Some of this work is detailed in a recent publication (Rogers et al., *Nucleic Acids Research* 2004 32(22):6595-6604).

TAT peptides as vectors for transfer of macromolecules into living cells.

Introduction.

Most biopolymers do not readily translocate across biological membranes. However, some proteins, such as transactivating factors and homeoproteins, have been shown to be capable of facilitating membrane translocation. This ability is associated with specific protein domains enriched with basic amino acids. Well known examples include Tat derived peptides, the third helix of the *antennapedia* homeodomain protein, and VP22. Translocation of peptides/proteins and nucleic acids has been achieved by covalent conjugation with cell penetrating peptides.

The use of peptides with both cell penetrating domains and “coupling” domains that permit non-covalent binding of cargo molecules to the peptide would markedly simplify the construction of translocation complexes and greatly enhance the flexibility and diversity of methods for translocation of macromolecules into a cell. We have designed such artificial peptide vectors, which contain a TAT peptide linked to an oligolysine adapter domain, able to electrostatically bind cargo macromolecules and will describe here their construction and use.

Materials and Methods

Cell Culture and Flow Cytometry Analysis

Hela cells and mouse LTK⁻ cells were grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, glutamine, penicillin and streptomycin. SK-BR-3 human adenocarcinoma cells (ATCC No. HTB-30) were grown in A-medium (McCoy 5A medium supplemented with 15% fetal bovine serum, glutamine, penicillin and streptomycin). Cells were harvested while in the exponential phase of growth, washed and resuspended in growth medium or Dulbecco's phosphate buffered saline (DPBS).

Flow cytometric analysis of cell subpopulations was performed with support of Rocco Carbone of the Yale Cancer Center Flow Cytometry Shared Resource, using FACS Vantage flow cytometer (Becton-Dickenson Immunocytometry Systems, San Jose, CA). The cells were excited at 488 nm. The FITC fluorescence was collected through 530/30 nm band pass filter. A minimum of ten thousand cells were interrogated in each sample. Analysis of data was performed using CellQuest software (Becton-Dickenson).

To detect fluorescent labeled molecules associated with cells, the cells were first harvested in DPBS, incubated with a fluorescent labeled molecule of interest at varied concentrations (e.g., oligonucleotide at 50 μ M mixed with 1×10^5 cells in 10 μ l DPBS), incubated for 15 minutes at room temperature, diluted with 200 μ l growth medium, and then analyzed by fluorescence activated cell sorting (FACS).

Peptides and Oligonucleotides

Peptides were prepared in Yale Keck Facility using Fmoc solid phase peptide synthesis techniques and purified by HPLC using a C18 reverse phase column. Peptides were dissolved at a concentration of 8 mM in 500 mM Hepes buffer (pH 7), then diluted with DPBS to appropriate concentrations before the experiment. Peptides were administered to the cells in concentrations ranging from 0-800 μ M. Oligonucleotides were prepared and purified in The Pathology Department DNA Synthesis Laboratory (Table 1).

Table 1

Peptides and oligonucleotides used in the work

Name	Sequence
Tat	G4YGRKKRRQR
K16	K16
TAK8	G4YGRKKRRQR3G4K8
TAK12	G4YGRKKRRQR3G4K12
TAK16	G4YGRKKRRQR3G4K16
F-Tat	FITC- G4YGRKKRRQR
F-K16	FITC-K16
F-TAK8	FITC-GYGRKKRRQR3G4K8
F-TAK12	FITC-G4YGRKKRRQR3G4K12
F-TAK16	FITC-G4YGRKKRRQR3G4K16
Cy3-O18	5'-Cy3-TATATGATGGTACCGCAG-'3-dT-5' (dT bipolar-oligonucleotide with inversion)
Fluor-O-24	FITC-TCAGAACTCACCTGTTAGACGCCA-3'-3-hydroxy-1-propyl
O-83-Fluor	TTGATAAGAGGTCATTTTTGCGATGGCTTAGAGCTTAATTGC TGAATCTGGTGCTGTAGCTCAACA TGTTTTAAATATGCAA-3'-FITC
Bio-O-22	biotin-CCAGACTGAGTATCTCCTATCA

DNA

Plasmid DNA pCEGF (5 kb, Clontech) contains a modified green fluorescent protein gene with enhanced cyan fluorescence, whose expression is under the control of a CMV promoter in mammalian cells. A 410 bp linear double stranded DNA fragment was made by PCR from the DRD2 gene (coordinates:11812-12222) and purified by electrophoresis. For transfection, cells were collected, washed in DPBS and resuspended in DPBS. 200 ng of plasmid DNA and TAK16 (final concentration 4-10 μ M) in 50 μ l DPBS were added. The cells were incubated for 30 min at 4 or 37°C, then transferred into 3 ml medium in 6 well plate (Falcon). For lipofectin-mediated transfection, 2×10^4 cells were incubated with 200 ng of plasmid DNA and lipofectin as directed by manufacturer (Life technology, Inc.), after which the cells were placed in full growth medium. After 2 days incubation cells were collected and analyzed by microscope with FITC and Aqua filters.

Fluorescence Microscopy

Fluorescent images were captured using commercial software (Perceptive Scientific Imaging, League City, TX), a Sensys Camera (Photometrix, Tucson, AZ), and an Olympus microscope with multiple excitation and emission filters (Chroma Technology, Battleboro, VT). The nuclear DNA was counter-stained by mounting in Vectashield[®] (Vector Laboratories, Burlingame, CA) containing 2 μ g/ml DAPI (4',6 diamidino-2-phenylindole).

Preparation of TAK16-Oligo-Avidin conjugates

Avidin-FITC in DPBS was mixed with Bio-O-22 in DPBS to concentration 12 μ M avidin and 8 μ M oligo and incubated 15 min at room temperature. 1.5 μ l of the mix was added to 2×10^5 Hela cells in 10 μ l DPBS, then 11.5 μ l 100 μ M TAK16 was added. The cells were incubated for 20 min at 37°C, washed in DPBS and analyzed by microscope.

Horseradish Peroxidase-Avidin (HPA) uptake

HPA in DPBS was mixed with Bio-O-22 in DPBS to concentration 8 μ M HPA and 8 μ M oligo and incubated 15 min. at room temperature. 2.5 μ l of the mix was added to 2×10^5 Hela cells in 10 μ l DPBS, then 12.5 μ l 100 μ M TAK16 in DPBS, incubated 30 min at 37 grad, wash and applied on slides. Cells on the slides were treated with 30 μ l peroxidase supressor to supress endogenous peroxidase, washed with DPBS, treated with 30 μ l Metal Enhanced DAB Substrate Working Solution, and after 15 min incubation at room temperature analized by microscope.

Results

Translocation of oligonucleotides with a fusion peptide: TAK16, but not Tat peptide facilitates oligonucleotide translocation through the cell membrane.

The segment of HIV TAT protein encompassing amino acids 47-57 (TAT peptide) has been shown to translocate across cellular lipid bilayer membranes. This process is reversible: TAT peptide can be easily washed out of the cells incubated in the medium without peptide (data not shown, also S.F.Dowdy, personal comm.).

We designed a fusion peptide, TAK16, which contains TAT cell penetrating domain linked through a short oligoglycine bridge to "adaptor", in this case-cationic domain containing

16 lysines (Tabl.1). In contrast to the TAT peptide, TAK16 has some striking differences that derive from its two-domain structure. TAK16 uptake is an order of magnitude higher than uptake of TAT peptide and is irreversible because of its higher affinity to intracellular polyanions. Unlike the diffuse staining of cells treated with the FITC-TAT peptide, FITC-TAK peptide gives rather a speckled fluorescence pattern.

Both TAT and TAK16 are accumulated in the cells very quickly - within less than one minute cells incubated in vitro with 100 μ M of TAT or TAK16 were saturated with peptide. Both peptides show the relatively low toxicity for the cells. We did not note any inhibition in cell growth in the presence up to 4 mM peptides.

Cationic Tat peptide with 8 basic amino acids seems to have low affinity to nucleic acids, and could not facilitate translocation of an oligonucleotide across a cell membrane. In contrast, the TAK16 peptide has a high affinity for DNA and facilitates DNA and RNA translocation into cells. The high efficiency of TAK16 was demonstrated using the Cy3-labeled 18-mer oligonucleotide (Fig.1). Fluorescence analyses of HeLa cells treated with both the Flu-TAK₁₆ and the Cy3-oligonucleotide showed that the Cy3 label (red) of the oligonucleotide was located in the cytoplasm as well as in the nucleus. Furthermore, the Cy3 (oligonucleotide) and FITC (Tat-K₁₆ peptide) signals did not necessarily co-localize within a cell.

Various derivatives of TAK16 were tested for their abilities to facilitate oligonucleotide translocation across cell membranes. The peptides tested included: K16 (G₄K₁₆); TAT (G₄YGRKKRRQR₃); TAK8 (G₄YGRKKRRQR₃G₄K₈); TAK12 (G₄YGRKKRRQR₃G₄K₁₂) and TAK16/TatK₁₆. HeLa cells (1×10^5) were incubated with the FITC-labeled 24-mer oligonucleotide, FITC-O-24, and various concentrations of the peptides and then analyzed by FACS. TAK16, which has the longest lysine adapter domain of the peptides used, was the most efficient domain for facilitating transport of oligonucleotide. Neither TAT, nor adapter domain itself, K16, were able to efficiently facilitate transport. The data also reveal that, compared to TAK16, TAK12 and TAK8 had decreased ability to facilitate transport of an oligonucleotide, proportionally to the decreased lengths of the adapter domain.

Next we determined whether TAK16 peptide could facilitate transport of an even larger oligonucleotide. Cells were incubated with TAK16 at 13.3 μ M and the fluorescein labeled 83-mer oligonucleotide, O-83-FITC at different concentrations. Cells were then subjected to FACS analysis or fluorescent microscopic analysis. The FACS analysis (Figure 3) showed that at a fixed concentration of TAK16, the amount of cell associated fluorescent intensity increased with increasing amounts of the FITC-O-83 to which the cells were exposed.

TAK16 mediated translocation of double-stranded DNA across cell membranes

Not only oligonucleotides, but also double-stranded (ds) DNA or plasmid DNA, could be transported into a cell. Specifically, we determined whether TAK16 could facilitate the transport of 400 base pair (bp) double-stranded DNA and a 5 kb circular plasmid DNA (pECFP; enhanced cyano fluorescent protein) into cells. The DNAs were labeled with Cy3 reagent (Materials and Methods). This protocol usually yields at least one label per ten nucleotides. HeLa cells were incubated with 400 ng of plasmid DNA or 60 ng of 400 bp DNA and with TAK16.

The results of this set of experiments are presented in the fluorescence micrographs of Figure 5. It indicates that plasmid DNA alone does not enter the cell in the absence of TAK16 (Figure 5A). However, when the cells were incubated with 400 bp dsDNA or plasmid DNA in the presence of TAK16 peptide, there was uptake of DNA associated fluorescence (Figure 5B,C). These data indicate that fusion peptides with adapter domains such as TAK16, facilitate

transport of large DNA molecules into cells, and uptake of large double stranded DNA molecules, both linear and circular, occurs in virtually 100% of the cells.

Plasmid DNA delivered into the cells by TAK16 vector maintained its biological activity. HeLa or SK-BR-3 cells were incubated with pECFP and TAK16 as described above. Controls included pECFP alone or pECFP combined with lipofectin (positive control). Plasmid transient and stable transfection was determined using expression of the plasmid markers, Enhanced Cyano Fluorescent Protein (ECFP) and NeoR. After 2 days incubation, transient transfection efficiency with TAK16 vector was much higher than for pECGF DNA alone, and about one third of the transfection with lipofectin. The same pattern was obtained for stable transfection when cells were incubated in the presence of G418 (Tabl.5).

Table 2.
pECGF transfection in HeLa cells.

Transfection efficiency of pCEGF DNA					
	No peptide	+TAT	+K ₁₆	+TAK16	lipofectin
transient transfection	$<2 \times 10^{-3}$	$<2 \times 10^{-3}$	$<2 \times 10^{-3}$	1.2×10^{-1}	3.1×10^{-1}
stable transfection	$<1 \times 10^{-6}$	$<1 \times 10^{-6}$	$<1 \times 10^{-6}$	3.5×10^{-5}	1.1×10^{-4}

Cells were transfected with pCEGF as described in Materials and Methods and incubated 2 days in growth medium for transient transfection or 2 weeks in growth medium with 750 ug/ml G416 for stable transfection.

TAK16 as vector for oligonucleotide-mediated mutagenesis.

TAK16 bound to triplex-forming oligonucleotide facilitates mutagenesis in murine cells. The model of mutagenesis contains murine fibroblast cell line with bacterial SupFG1 gene integrated into the eukaryotic genome and the use of a 30-mer purine-rich oligonucleotide able to bind and form a mutagenic triplex structure within the SupFG1 gene.

Cells were treated with two triplex forming oligonucleotides: AG13 and AG30 and different delivery systems. In these experiments, TAK16 was even more efficient as a vector than the Antennapedia (ANTP) -s-s-oligo complex or the lipid delivery system (Gene Porter) (Tabl.6).

Table 6.
Chromosomal Mutations Induced in SupG1 Gene by
Triplex- Forming Oligonucleotides

Treatment	Mutations /Total Plaques	Mutation Frequency (x 10 ⁻⁵)
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Control	4/52,175	7
AG13	3/37,200	8
AG13 + Gene Porter	2/23,000	9
AG13-S-S-ANTP	5/19,278	25
AG13 + TAK16	19/48,000	40
AG30 + Gene Porter	2/11,400	21
AG30 + TAK16	11/30,613	36

AG13 = 5'-AGGAA8-propylamine-3'

AG30 = 5'-AGGAAG8TG GTG5AG5AG-propylamine-3'

Fig.6:Avidin-FITC uptake mediated by TAK16-oligo vector.
(1)Control-no oligo, (2)avidin-FITC uptake (FITC).

Fig.7. HPA uptake mediated by TAK16 –oligo vector.
(1) Control- no oligi, (2) HPA uptake(brown staining).

The effects of cell-penetrating transport peptides, derived from the third helix of the homeodomain of antennapedia (Antp) and from x, on the biological activity of Triplex-forming oligonucleotides (TFOs) were measured. Assays for gene-targeted mutagenesis utilizing TFOs covalently linked to Antp demonstrated a 20-fold increase in mutation frequency when compared with 'naked' oligonucleotides. There was no increase above background in mutation frequency when Antp by itself was added to the cells or when Antp was linked to mixed or scrambled sequence control oligonucleotides. In addition, the TFO-peptide conjugates increased the mutation frequency of the target gene, and not the control gene, in a dose-responsive manner. Confocal microscopy using labeled oligonucleotides indicated increased cellular uptake of TFOs when linked to Antp, consistent with the gene-targeting data. These results suggest that peptide conjugation may enhance intranuclear delivery of reagents designed to bind to chromosomal DNA.

Peptides

Antp (RQIKIWFQNRRMKWKK) was synthesized by SynPep (Dublin, CA) or purchased from Qbiogene (Carlsbad, CA). The peptide was activated with a pyridyl disulfide function at its N-terminal end, to facilitate conjugation to the oligonucleotide. The peptide was high-performance liquid chromatography (HPLC) purified and analyzed by mass spectrometry.

Oligonucleotides

Oligonucleotides were synthesized by the Midland Certified Reagent Company (Midland, TX), using cyanoethyl phosphoramidite chemistry. After removal of the protecting groups by hydrolysis with concentrated ammonium hydroxide, the product was purified by reverse-phase HPLC. The ammonium salt form of the oligonucleotide was dissolved in distilled water and further purified using a NAP5 Sephadex G25 filtration column (Amersham Pharmacia Biotech). To allow coupling to the peptide, the TFO, AG30, 5'-AGG AAG GGG GGG GTG GTG GGG GAG GGG GAG (CH₂)₃ S-S-3' was synthesized with a 3'-thiol group. This group was introduced

by utilizing a 3'-thiol-modifier C3 CPG (Glen Research) as the solid support. The scrambled control oligonucleotide, SCR30, 5'-GGA GGA GTG GAG GGG AGT GAG GGG GGG GGG (CH₂)₃ S-S-3', was synthesized using the same solid support, and used for comparison with AG30 in the mutagenesis experiments. The 3'-amino-modifier C7 CPG (Glen Research) was used to synthesize the unconjugated AG30 with a 3'-amine to prevent nuclease degradation. Labeled AG30 was synthesized with 5'-rhodamine and 3'-propyldisulfide modifications using rhodamine phosphoramidite and the 3'-thiol-modifier C3 S-S CPG, respectively. The labeled oligonucleotide was purified by anion-exchange HPLC. The 5'-rhodamine labeled AG30 was used in the determination of the cellular localization of the TFO-peptide conjugate. In addition, the control oligonucleotide, MIX30, 5'-AGT CAG TCA GTC AGT CAG TCA GTC AGT CAG (CH₂)₃ S-S-3' was also synthesized with 5'-rhodamine and 3'-propyldisulfide modifications and used for comparison with AG30 in the microscopy and mutagenesis experiments.

The TFO, AG13, with the following sequence 5'-AGG AAG GGG GGG G (CH₂)₃ S-S-3', was synthesized at Isis Pharmaceuticals (Carlsbad, CA). The TFO was synthesized using standard phosphoramidite chemistry with a 3'-thiol modifier, C3 S-S CPG (Glen Research) for peptide conjugation. Following deprotection by hydrolysis using concentrated aqueous ammonium hydroxide, the product was purified by reverse-phase HPLC. The oligonucleotide in the ammonium salt form was desalted by reverse-phase HPLC. The unconjugated AG13 was synthesized with a 3'-methoxyethyl modification at the 3' end to ensure nuclease resistance.

Synthesis of TFO-peptide conjugates

The 3'-thiol modified TFO, AG30 (2.38×10^{-8} moles, 236.8 μ g), was dissolved in 250 μ l of 0.1 M DTT and the reduction of the thiol group was allowed to proceed overnight at 37°C. The solution was then applied to a NAP5 filtration column pre-equilibrated with degassed water. The reduced oligonucleotide was eluted under gravity with 1 ml of degassed water directly into a siliconized Eppendorf tube containing a solution of activated Antp (Penetratin 1, Qbiogene) (65 μ l). If precipitation was observed, a small amount of methanol was added dropwise, and the reaction mixed by vortexing. The reaction mixture was heated at 65°C for 15 min in a tightly sealed tube, in order to eliminate the formation of secondary structures and prevent further precipitation. The reaction was incubated for an additional hour at 37°C. If required, the methanol was evaporated using a centrifugal evaporator following completion of the incubation period. The coupling reaction was monitored by means of SDS-PAGE, achieving a coupling yield of more than 95%. The overall synthetic strategy was similar to the procedure supplied by the manufacturer (Qbiogene).

The synthetic strategy used to synthesize the AG13-peptide conjugate was similar to that of the AG30-Antp, however, in this case, the oligonucleotide was activated. The deprotected 3'-thiol modified TFO, AG13 (50 OD), was dissolved in 1 ml of 0.5 M NaHCO₃/Na₂CO₃ buffer (pH 9), and 1 M DTT was added to give a final concentration of 0.25 M. The reduction of the thiol was allowed to proceed at room temperature for 2 h. Residual DTT was removed by RP-HPLC using a 0–40% gradient of 0.1 M NH₄OAc over 60 min in 80% CH₃CN. The purified, reduced AG13 was collected directly into a flask containing 2 mg of 2,2'-dipyridyl disulfide (Aldrich) in 500 μ l of CH₃CN and 15 μ l of *N*-ethyldiisopropylamine [EtN(iPr)₂]. After 2 h at room temperature, the reaction was concentrated in vacuum, followed by removal of excess 2,2'-dipyridyl disulfide by extraction with dichloroethane. The 3'-(2-pyridyl disulfide) derivative of AG13 was further

purified by RP-HPLC. The amount of purified, activated oligonucleotide was determined spectrophotometrically.

Activated AG13 was dissolved in a degassed solution of 0.1 M KCl, 1 M urea, 0.1 M NH₄OAc, 20% acetonitrile and 1 μ M EtN(iPr)₂. Under gentle mixing, Antp (10 mg), dissolved in degassed water, was added slowly to the solution of activated oligonucleotide under argon. The reaction was incubated for 2 h under argon at room temperature. The resulting conjugate was purified by ion-exchange HPLC on a Mono Q column using a 0–50% gradient of 1.5 M NaBr established over 60 min in 0.1 M NH₄OAc, 2 M urea, 30% CH₃CN. Yield 85%. The amount of AG13–Antp conjugate was determined spectrophotometrically based on molar absorption coefficients. The purity of the conjugate and verification of its composition was determined by RP-HPLC and 15% denaturing gel.

Cells and mutagenesis assay

C127 cells (CRL-1616), derived from a mammary tumor of an RIII mouse, were obtained from the American Type Culture Collection (Manassas, VA). The cells were transfected with λ supFG1 DNA using cationic lipids and selected in the presence of the neomycin analog G418 at 0.8 mg/ml (Life Technologies, Gaithersburg, MD). The established cell line, AV16, containing >15 copies of the λ supFG1 shuttle vector, was maintained in medium (DMEM supplemented with 10% fetal calf serum) containing G418 at 0.8 mg/ml. Peptide conjugates were diluted (to a final concentration of 2 μ M) in 2 ml of serum-free media before addition to the cells. Prior to the addition of the peptide conjugates, the cells were washed several times to eliminate the possibility of the peptide binding to dying cells and fragments of double-stranded DNA, which would lead to a reduction in the interaction with phospholipids. AV16 cells, at 70% confluence, were washed with serum-free medium, treated with the TFO–peptide mixture (or each component alone) and incubated at 37°C. Following a five hr incubation, the medium volume and serum concentration were adjusted to that of normal growth conditions. The cells were collected for shuttle vector rescue and analyzed 2–4 days following treatment. Genomic DNA was isolated from the cells as described previously. The DNA was incubated with *in vitro* packing extracts for λ vector rescue from the genomic DNA and reporter gene analysis. Each experiment was repeated three times, and standard errors were calculated for the mutation frequency values (indicated by the error bars in the respective figures).

Microscopy

AV16 cells were plated onto glass bottom culture dishes (MatTek Corp., Ashland, MA) and grown to 70% confluency. Before addition to the cells, the rhodamine labeled oligonucleotide–peptide hybrids, AG30–Antp and MIX30–Antp, were heated at 55°C for 10 min and cooled to room temperature. After the cells were washed with serum-free media, diluted rhodamine labeled conjugate was added at a final concentration of 2 μ M. For direct detection of conjugate uptake, the cells were washed extensively with serum-free media after the designated incubation period at 37°C and live cells were immediately analyzed using a Zeiss LSM 510 META confocal laser microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY). Confocal microscope images of unfixed AV16 cells treated with either rhodamine labeled TFO–peptide conjugate were taken at 63x using Cy3 filter sets. For purpose of consistency, all images were focused on the nuclear membrane, with an exposure time of 1.6 s.

Induced chromosomal mutations

One major obstacle in the quest to use oligonucleotides as therapeutic agents for targeted gene therapy is their limited ability to cross cell membranes. To investigate the ability of translocating peptides to improve intracellular delivery of TFOs to chromosomal sites, an assay for targeted mutagenesis in mammalian cells was used. Unlike the conventional approaches used to evaluate antisense activity, this assay does not rely on the inhibition of gene expression, where non-specific toxic effects can be confused with antisense activity. Instead, the assay has a quantitative readout for chromosomal gene targeting. We utilized a mouse cell line (AV16), containing multiple copies of λ supFG1 shuttle vector DNA in a chromosomal locus. The *supFG1* reporter gene contains a polypurine/polypyrimidine target site for third strand binding by either AG30 or AG13. Using packaging extracts, the vector DNA can be isolated from mouse genomic DNA into phage particles and subsequently analyzed for induced mutations. *SupFG1* encodes an amber suppressor tRNA whose function can be scored in indicator bacteria. A targeted mutation in the *supFG1* gene will result in the production of a white plaque upon shuttle vector rescue, in contrast to the blue plaques produced by functional *supFG1* genes. This is a useful system for evaluating the delivery of oligonucleotides to the nucleus, because site-directed mutagenesis induced by TFOs at the *supFG1* gene has been established both *in vitro* and *in vivo*.

Cells were incubated for 5 h with the peptide conjugates to allow cellular uptake. In order to provide an opportunity for triplex formation and mutation induction via repair and replication, the cells were harvested and the vector DNA was isolated for genetic analysis following an additional 48 h. Each experiment was repeated three times, and standard errors were calculated for the mutation frequency values. The results indicate that treatment of the cells with the TFO-peptide conjugates induced mutations at higher frequencies compared with treatment with the TFO alone. The AG30-Antp hybrid molecule induced a 20-fold increase in mutation frequency when compared with direct addition of the TFO to the cell culture medium. The lack of induction when AG30 and Antp were simply mixed together before addition to the cell demonstrates the need for covalent linkage of the TFO to the peptide to achieve intracellular activity. Addition of Antp, by itself, to the cells did not result in induction of mutagenesis, providing evidence that the increased mutagenesis was dependent on improved TFO delivery and was not due to any peptide-induced mutations. The inability of the control oligonucleotide-peptide conjugate, MIX30-Antp, to induce mutations above background provides evidence to support a mechanism of induced mutagenesis dependent upon the binding of the TFO to its specific target site. This mechanism is further supported by the inability of the G-rich scrambled sequence control oligonucleotide-peptide conjugate, SCR30-Antp, to induce mutations above background. Transfection of AG30 using cationic lipids resulted in a mutation frequency of 31×10^{-5} , a 12-fold increase when compared to the addition of 'naked' AG30 alone to the cells. However, when the two methods of TFO transfection are compared, AG30-Antp gave mutation frequencies that were 2-fold higher than the frequency obtained when cationic lipids were used.

Sequence analysis of the AG30-Antp induced mutations revealed base substitutions and single base pair insertion and deletion mutations precisely within the TFO-binding site. Because the majority of mutants analyzed were located in the triplex-binding site, the sequencing data provide further evidence that the mutations were TFO-induced and site-specific. To provide additional evidence to support a mechanism of induced mutagenesis dependent on TFO binding to a specific chromosomal target site, and to confirm specificity of gene targeting, we screened the *lacII* gene in the same vector for possible non-specific induction of mutagenesis by the AG30-

Antp peptide conjugate. The *cII* gene does not contain the TFO-binding site. In untreated AV16 cells, a background frequency of mutations in the *cII* gene of 6×10^{-5} (3/51 633) was observed. In cells treated with 2 μ M AG30–Antp, a mutation frequency of 7×10^{-5} (6/91 800) was obtained for the *cII* gene, essentially at background. Hence, there is no non-specific mutagenic effect of the peptide conjugate in the absence of potential triplex formation. Taken together, the analysis of the pattern of mutations and the evaluation of the non-targeted *cII* gene show that the increase in mutation frequency in *supFG1* is a result of a specific and site-directed effect of the TFO–peptide conjugate and TFO binding to the *supFG1* chromosomal target site.

We also tested the ability of an Antp conjugate to increase the biological activity of a TFO with weaker binding affinity to the *supFG1* target site. AG13 was designed to bind to the first 13 bases in the 30 bp triplex-binding site in the *supFG1* gene, and binds with an affinity (equilibrium dissociation constant) of $\sim 10^{-8}$ M versus $\sim 10^{-9}$ M for AG30. We treated AV16 mouse cells with AG13 using both peptide conjugation and cationic lipid co-mixture. Conjugation of AG13 to the carrier peptide Antp resulted in a 6-fold increase in mutation frequency (30×10^{-5}) compared to direct addition of the AG13 alone to the cell culture medium. Interestingly, when transfection with cationic lipids was used, no significant increase in mutation frequency above AG13 alone was observed. Hence, the Antp conjugate conferred biological activity on AG13 that was not achieved by cationic lipid transfection. The ability of Antp conjugation to increase the effectiveness of the AG13 TFO further suggests the importance of TFO delivery in achieving bioactivity, especially with TFOs of intermediate binding affinity. The results also suggest that some polypurine sites that may be sub-optimal for TFO binding due either to reduced length or sequence inversions may still be targetable by peptide-conjugated TFOs. Sequence analysis of the AG13–Antp induced mutations also revealed base substitutions, insertions and deletions precisely within the TFO-binding site. These sequencing data demonstrate that the reduced binding affinity of the TFO does not affect the specificity of gene targeting. To confirm specificity of gene targeting, we also screened the *lacII* gene in the same vector for possible non-specific induction of mutagenesis by the AG13–Antp conjugate. In cells treated with 2 μ M AG13–Antp, a mutation frequency of 9×10^{-5} , was obtained essentially at background. As a result, one can conclude that the increase in mutagenesis observed with the AG13–Antp conjugate can be attributed to improved delivery.

To investigate further the ability of the peptide to improve the biological activity of TFOs, dose–response experiments demonstrated that the induced mutation frequency increased with increasing concentrations of the AG30–Antp conjugate. A mutation frequency of 47×10^{-5} was observed when AV16 cells were treated with 0.1 μ M of AG30–Antp, while a frequency of 86×10^{-5} was obtained with an increased concentration of 5 μ M. Similar results were observed in the case of AG13–Antp. As the concentration of conjugate administered increased, an increase in mutation frequency was noted, ranging from 24×10^{-5} to 58×10^{-5} with treatments of 0.1–5 μ M, respectively.

Microscopy

It has been shown that peptides bind strongly to the cell membrane and remain associated with cells even after repeated washings. As a result, FACS analysis cannot be reliably used to evaluate uptake unless a protease digestion step of the adsorbed proteins is included in the protocol. Cellular uptake of the AG30–Antp conjugate was instead evaluated using confocal microscopy. Because it has been demonstrated that cell fixation, even under mild conditions, leads to the artifactual uptake of transport peptides, we chose to perform microscopy on live unfixed cells.

This technique allowed us to visually demonstrate a difference in the uptake of labeled-TFO and labeled TFO–Antp.

The rhodamine-labeled AG30–Antp and the rhodamine-labeled AG30 were incubated with AV16 cells at 37°C for periods of 5 and 24 h, after which the cells were examined by confocal microscopy. Surprisingly, rhod–AG30 exhibited significantly more uptake than would be expected by passive diffusion, with uneven distribution of the oligonucleotide throughout the cell (Fig. 1). The majority of the fluorescence is observed in the cytoplasm, with punctate distribution in the area surrounding the nucleus. However, there was some detectable uptake into the nucleus. The intensity of fluorescence did not increase with time. In fact, after 24 h, there was an apparent decrease in the intensity of punctate foci in the area surrounding the nucleus (Fig 1B) Rhodamine–AG30 was also transfected into the cell using cationic lipids. This method of transfection resulted in punctate distribution of fluorescence in the area surrounding the nucleus, with negligible staining of the nucleus (Fig 1C). After 24 h, the intensity of the rhodamine increased, but there was still no significant uptake in the nucleus (Fig 1D).

Figure 1. Internalization of rhodamine-labeled AG30–Antp conjugate by AV16 mouse cells. Cells were incubated with either rhodamine–AG30 (**A** and **B**), rhodamine–AG30 mixed with cationic lipids (CL) (**C** and **D**) or rhodamine–AG30–Antp (**E** and **F**) at a concentration of 2 μ M for 5 or 24 h. Unfixed cells were examined by confocal microscopy.

The AG30–Antp conjugate was more evenly distributed within the cells, with less difference between the nucleus and cytoplasm (Fig. 1E). Both diffuse and punctate cytoplasmic fluorescence was observed. After 24 h, the accumulation of labeled AG30–Antp increased, with higher levels of punctate cytoplasmic fluorescence (Fig. 1F). Some fluorescence was observed on the cell surface, which can be attributed to the accumulation of oligonucleotide–peptide conjugate. This may be caused by the formation of secondary structures, due to the G-rich content of AG30. (The conjugate was heated at 55°C for 5 min before use in uptake experiments to try to minimize this phenomenon.) Overall, comparison of the images reveals that the rhodamine signal accumulated in the cells to varying extents in all cases. However, the intensity of intracellular fluorescence is greater for the AG30–Antp conjugate than for the oligonucleotide

alone. In addition, the rhodamine-labeled AG30–Antp conjugate showed a less punctate and more diffuse cellular distribution than did the rhodamine-labeled unconjugated AG30.

Nonetheless, the uptake of AG30 without the aid of any special transfection method beyond addition to the culture medium is noteworthy. Other microscopy based analyses of oligonucleotide uptake have revealed minimal uptake in the absence of transfection. To investigate whether the uptake observed with AG30 might be due to its G-rich content, a rhodamine-labeled oligonucleotide with a balanced base composition, MIX30, was incubated with AV16 cells for 5 h. Uptake of MIX30 was examined using confocal microscopy, and no detectable intracellular fluorescence was observed (Figure 2A), consistent with other published reports. Some uptake of rhod–MIX30 was eventually detectable after 24 h (Figure 2B), but at intensities similar to what was observed with rhod–AG30 after only 5 h (Figure 1B).

Figure 2. Internalization of rhodamine labeled MIX30–Antp conjugate in AV16 mouse cells. Cells were incubated with either rhodamine–MIX30 (**A** and **B**) or rhodamine MIX30–Antp (**C** and **D**) for 5 or 24 h, as indicated and examined by confocal microscopy without prior fixation.

The distribution of uptake in the case of MIX30 at 24 h was also punctate in the region surrounding the nucleus, suggesting endocytosis as a possible mechanism of uptake. In contrast, when rhod–MIX30 was conjugated to Antp and incubated for 5 h with AV16 cells, there was substantial cellular uptake, even more than the AG30–Antp conjugate (Figure 2C). In addition, the distribution of rhod–MIX30 within the cells was mostly nuclear, with lesser intensity in the cytoplasm (Figure 2C). After a 24 h incubation period, the fluorescence distribution was the same (Figure 2D). Hence, although Antp conjugation enhanced the overall cellular uptake of both AG30 and MIX30, a much higher proportion of intranuclear uptake was achieved with MIX30. These studies suggest that the characteristics of the oligonucleotide cargo linked to a cell-penetrating peptide can affect the efficiency of intracellular delivery and the pattern of

intracellular distribution. However, one caveat to keep in mind is that this cargo-dependent effect was observed in just one cell line (AV16), and may not be generalizable to all cell lines.