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Transport of phosphorothioate oligonucleotides in kidney: Implications for molecular therapy

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Transport of phosphorothioate oligonucleotides in the kidney: Implications for molecular therapy. The systemic administration of phosphorothioated antisense oligonucleotides has been demonstrated to be an effective strategy for the control of gene expression. Because previous studies have suggested both hepatic and renal accumulation of systemically administered oligonucleotides, we explored whether the kidney might be a site of free DNA transport. [^{32}P]-phosphorothioate oligonucleotides (20 mers) were excreted in urine but cleared at only 30% of glomerular filtration rate. Plasma clearance of the label was very rapid ($t_{1/2}$ ~5 min) but the half life of labeled S-deoxynucleotide excreted in urine was much slower (28 min). Infused oligonucleotide appeared in urine with little degradation. By autoradiography of renal tissue, labeled antisense oligonucleotides appeared within Bowman's capsule and the proximal tubule lumen. DNA was detected in association with brush border membrane and within tubular epithelial cells. Brush border membrane preparations from rat kidney contained oligonucleotide binding proteins as determined by gel mobility shift and UV cross linking assays. Because renal epithelial cells efficiently take up phosphorothioate oligonucleotides without apparent degradation, the kidney appears to be an excellent target for site-directed antisense therapy, but may be a site of antisense toxicity as well.

Short oligonucleotides with mRNA complementarity can specifically interfere with stability and translation of mRNAs [reviewed in 1–5]. The hybridization of antisense phosphorothioate oligodeoxynucleotides to mRNA activates cellular RNAse H activity, leading to degradation of targeted mRNA species [1, 6, 7]. The ability to design specific antisense oligomers based on various mRNA target sequences has led to the emergence of numerous studies *in vitro* [8–16], and *in vivo* [17–19] which demonstrate the utility of antisense oligonucleotides to inhibit specific gene expression. This approach may have broad application in the treatment of diseases such as AIDS, cancer, progressive renal failure as well as various other disease states that involve the expression of cellular and/or viral gene products [5, 20–23]. The stabilization of oligonucleotide by various chemical [6, 24–26] and structural [27] modifications has furthered the potential use of antisense therapy. Methyl phosphonates, phosphorothioates, and other modifications improve the half-life of antisense molecules intra- and extracellularly, thereby allowing gene-specific suppression at pharmacological doses [20–22, 28, 29]. The phosphorothioate oligonucleotides are particularly attractive as therapeutic agents, since these modifications retain solubility and RNAse H activation properties [7, reviewed in 2].

Modified oligodeoxyribonucleotides bind to the surface of cells and are readily internalized by a mechanism that appears to be receptor mediated [29–33]. The efficacy of antisense therapy may depend on the ability of antisense to bind to the cell surface and the fraction of antisense molecules which can escape trapping in the endosomal compartment. Antisense oligonucleotides that can enter the cell are rapidly transported to the nucleus [34–36]. Keratinocytes are notably efficient in this regard, without apparent endosomal accumulation of oligonucleotides [37]. Although the mechanism of uptake has not been established, several studies have identified cell surface nucleic acid binding proteins which may serve as receptors for nucleic acid [30, 31, 33, 38–40].

Distribution of antisense oligonucleotide *in vivo* is organ-dependent, with predominant accumulation in liver and kidney [41–43]. While localization in these sites appears to reflect metabolic elimination and excretion, this preferential distribution might reflect efficient mechanisms for binding and internalization of nucleic acids. Our results support this latter hypothesis, suggesting that accumulation of oligonucleotides in the kidney is mediated, in part, by a transport mechanism.

Methods

Synthesis and labeling of phosphorothioate oligonucleotides

Phosphorothioate antisense (S)-oligodeoxynucleotides targeting the Sp1 and TAR sequence found in all HIV-1 transcripts were synthesized using an Applied Biosystems 392 synthesizer, the sulfurization cycle, and TETD sulfurizing agent as recommended by the manufacturer. TAR and Sp1 sequences are dTCC-CAG-GCT-CAG-ATC-TGG-TC and dGAG-TTG-TGG-CCT-GGG-CGG-GAC-TGG-GGA-GTG-GC, respectively. Oligonucleotides (100 ng to 2 μg) were labeled in 50 μl reactions with terminal transferase (Boehringer Mannheim Biochemicals) and 50 to 150 μCi α-[^{32}P]-ddATP (Amersham). Alternatively, oligonucleotides were 5′-labeled with γ-[^{32}P]-ATP to exclude differences in renal handling based on the position of the phosphate label. Labeled oligonucleotides were purified on 15% denaturing polyacrylamide

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gels, eluted in ammonium hydroxide, dried, resuspended in water, and passed over a G-25 spin column equilibrated in PBS.

Tissue distribution of oligonucleotides

To study *in vivo* localization of phosphorothioate antisense, we administered [^{32}P]-labeled phosphorothioate oligonucleotides to FVB/N mice and studied the appearance of radioactivity in various organs. A 20 mer phosphorothioate modified antisense with complementarity to the HIV-1 TAR element was 3′ labeled and injected intravenously into mice. Mice were sacrificed and organs collected at 15, 30, 60, 120, 240, and 360 minutes. Radioactivity was measured by liquid scintillation counting of aliquots of homogenized tissues.

Renal clearance of oligonucleotides

Renal clearance studies were performed in mice by standard methods [44]. Briefly, mice were anaesthetized with 0.04 mg/gram pentobarbital and a polyethylene catheter (PE-90) was placed in the trachea to facilitate spontaneous ventilation. For the purposes of intravenous infusion, intermittent blood sampling, and mean arterial pressure monitoring, the left carotid artery and jugular vein were cannulated with polyethylene catheters (PE-10). Surgical blood loss was replaced by an infusion of normal saline (2% total body wt). [^{3}H]-inulin was administered first as a priming dose followed by an infusion in normal saline (0.25 μl/min/g body wt). The bladder was cannulated via a suprapubic incision with a PE-50 catheter. After 30 minutes of equilibration, [^{32}P]-labeled phosphorothioate oligonucleotides (2 to 5 × 10^5 cpm) were injected intravenously. Urine samples were collected during two consecutive 30-minute clearance periods and blood samples were taken at the midpoint of each 30-minute clearance period. Renal clearance after administration of unlabeled oligonucleotide was also determined. Following two baseline collections, an unlabeled bolus (0.33 mg TAR oligonucleotide) was delivered by a continuous infusion of 10 μg/min. Ten minutes after starting this infusion a second injection of [^{32}P] labeled oligonucleotide was given. Urine was collected for two additional 30-minute clearance periods and blood samples were collected at the midpoint of each clearance period. Radioactivities ([^{3}H]-inulin and [^{32}P] labeled S-oligonucleotide) were measured in aliquots of plasma and urine by liquid scintillation (Beckman) with appropriate discriminator windows set to minimize cross contamination. The isotope clearance was calculated as:

$$C_{isotope} = [Urine_{isotope}]V_{urine}/[Plasma_{isotope}]$$

Fractional excretion was determined by the following ratio:

$$C_{[^{32}P]\text{-oligo}} / C_{[^{3}H]\text{-inulin}}$$

Labeled phosphorothioate oligonucleotides in urine samples were analyzed by electrophoresis on 15% acrylamide denaturing gels.

Adhesion to whole blood cells

Whole blood was collected from mice and anticoagulated with either heparin or EDTA. EDTA or heparin-treated blood was then divided into 2 aliquots; in one sample from each, cells were washed three times in PBS and resuspended in the same volume as the plasma containing samples. To each of the four samples, 100,000 cpm of 3′ end-labeled oligonucleotide were added and allowed to incubate at room temperature for one hour. After one hour, cells were pelleted and the supernatant counted. Cells were washed twice in PBS, and the final cell pellet was resuspended in PBS and counted in liquid scintillation fluid.

Histology and autoradiography

FVB/N mice were injected intravenously with [^{35}S]-phosphorothioate TAR oligonucleotide labeled at internal sulfur substitutions [45]. Internally labeled TAR phosphorothioate oligonucleotide (approximately 700,000 cpm) was injected intravenously into FVB/N mice. Kidneys were harvested at the times indicated and histology sections were prepared by standard methods. Kidneys were fixed in neutral buffered formalin and embedded in paraffin. Three micron sections were coated with NTB2 emulsion (Kodak, Rochester, New York, USA), exposed in the dark at 4°C for one week and developed and fixed using Kodak chemicals by standard procedures. The tissue was counterstained with toluidine blue and photographed using a darkfield equipped microscope (Olympus, Tokyo Japan).

Purification of oligonucleotide binding proteins from brush border membranes

Membrane vesicles from renal brush border were purified as previously described [46]. Essentially, kidneys were collected from male Sprague-Dawley rats and placed immediately in cold mannitol buffer (10 mM mannitol, 2 mM Tris-HCl pH 7.1). Outer cortical tissue was harvested, weighed, suspended in 10 volumes mannitol buffer and homogenized. $CaCl_2$ was added to a final concentration of 10 mM and incubated on ice for 15 minutes. The mixture was diluted 1:1 wt/mannitol buffer containing 10 mM $CaCl_2$ and centrifuged at 15,000 × g for 12 minutes. The pellet was resuspended in mannitol buffer (150 ml) using a glass Teflon homogenizer. $CaCl_2$ was again added to a concentration of 10 mM and the mixture was incubated 15 minutes on ice. The mixture was then centrifuged at 15,000 × g for 12 minutes. The pellet was subjected to 3 resuspension/centrifugation (48,000 × g) cycles. Enrichment of brush border membrane proteins in the final suspension was confirmed by alkaline phosphatase activity of fractions [46]. The suspension (10 ml, approximately 0.5 mg protein/ml) was mixed with 5′-biotinylated-phosphorothioate oligonucleotide antisense immobilized on avidin-agarose (250 μl of affinity matrix containing approximately 150 μg of biotinylated oligonucleotide). Incubation was carried out at 4°C for 30 minutes. Beads were then washed three times in buffer. Binding proteins were eluted with 0.5 ml buffer containing 1.0 M KCl, 20 mM Tris 7.5, 1 mM EDTA, 1 mM DTT, 200 μg/ml BSA. Eluate was concentrated approximately 10-fold by centrifugal filtration. As a control, brush border membrane vesicles were incubated as described above with avidin-agarose-biotin matrix that lacked phosphorothioate oligonucleotides.

Electrophoretic mobility shift and UV cross linking assay

Proteins were prepared as above and incubated with the labeled S-oligodeoxynucleotides for 20 minutes at room temperature under conditions previously described [47]. Purified DNA binding proteins (10 to 15 μl concentrated eluate) were incubated in a standard gel shift reaction mixture (400 mM NaCl, 10 μg/ml polydI-dC and 10% glycerol) with α-^{32}P labeled oligonucleotide (10,000 cpm/reaction). Glycerol bromphenol blue dye was added

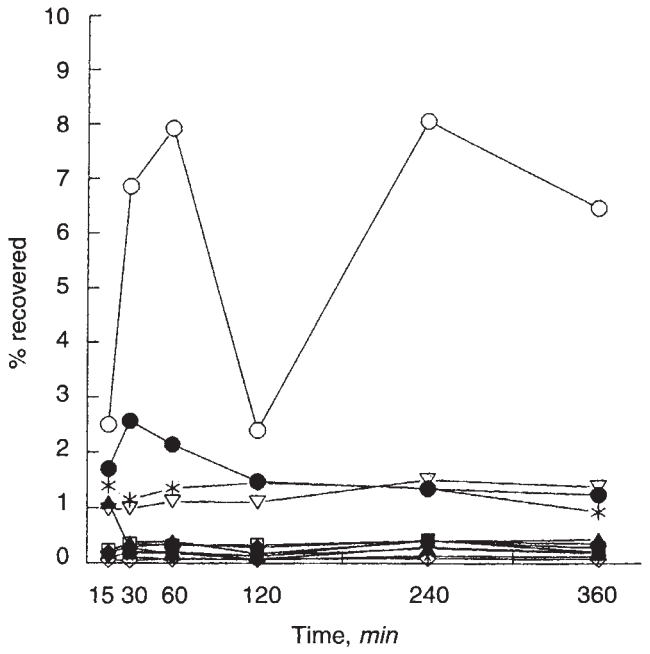

Fig. 1. *Organ distribution of a [^{32}P]-TAR oligonucleotide over time.* Symbols are: (○) liver; (●) kidney; (▲) fat; (△) spleen; (▼) lymph node; (◆) heart; (▽) muscle; (◇) brain; (□) lung; (×) testis; (✳) gut; (□) salivary gland. The majority of recovered oligonucleotide was found in liver and kidney. Methods: Six mice each received an intravenous injection (tail vein) of approximately 5.0×10^5 cpm of [^{32}P]-TAR in a volume of 300 μl. At each time point (15 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr) one animal was sacrificed and organs were isolated. Organs were harvested, homogenized, and counted by liquid scintillation. Data are expressed as percent of total injected counts.

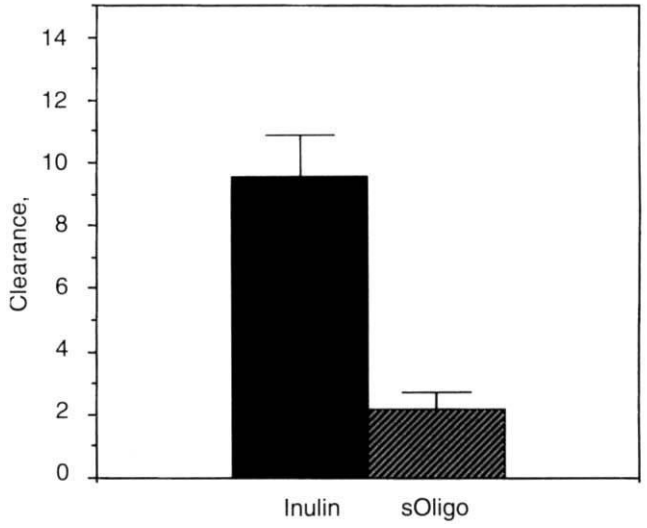

Fig. 2. *Clearances of inulin and phosphorothioate oligonucleotides (TAR) in vivo, determined simultaneously in anaesthetized mice.* The clearance of inulin in adult mice was 9.5 ± 1.5 ml/min/kg, the clearance of oligonucleotide was 2.0 ± 0.5 ml/min/kg. The fractional excretion of DNA (FE_{DNA}) was 0.30. Concentrations of [^{3}H]-inulin and [^{32}P]-labeled S-oligonucleotide in plasma and urine were determined in a liquid scintillation counter. Clearance and fractional excretions were calculated using standard formulae.

and samples were subjected to electrophoresis on a 6% polyacrylamide gel containing 3% glycerol in 0.5X TBE buffer, dried, and exposed to X-ray film. For UV cross linking analysis, binding reactions were UV irradiated approximately two inches from source for 20 minutes at 254 nm (1200 joules/min) in a room temperature bath. SDS sample buffer was added and the samples were subjected to electrophoresis on SDS-polyacrylamide (12%) gel.

Results

The kidney and the liver have previously been demonstrated to be major sites of antisense accumulation [41–43, 48]. To confirm this observation, antisense TAR S-oligodeoxynucleotide (20 mer) was injected intravenously and organs collected and counted at various times. Labeled oligonucleotides localized predominantly to liver and kidney (Fig. 1). Maximum renal tissue levels of oligodeoxynucleotides were detected within 30 minutes whereas maximum levels of counts were detected in liver after 60 minutes. In liver, with the exception of the single animal at 120 minutes, counts were sustained for the entire period of study (6 hr). In kidney, however, after the initial peak in counts at 30 minutes, there was a slow decline in the accumulation of radioactivity. By two hours, total counts in renal tissue were equivalent to muscle and gut, the two additional tissues that appeared to have significant uptake of phosphorothioated oligonucleotides.

As shown in Figure 2, inulin clearance or glomerular filtration rate was normal in these mice (approximately 9 ml/min/kg). The clearance of phosphorothioate oligonucleotides, however, was significantly less, approximately 30% of inulin clearance. Similar results were obtained whether oligonucleotides were labeled at the 5′ or 3′ end or whether random nucleotide sequences were utilized (data not shown). When unlabeled competitor oligonucleotide was administered, the fractional excretion increased from approximately 30% to 50% in two experiments suggesting displaceable binding occurs at some site. To examine the possibility of an intravascular cellular site, we examined binding of labeled oligonucleotides to heparinized whole blood. Fewer than 5% of labeled oligonucleotides could be precipitated by heparinized whole blood.

The decay curves for plasma and urine [^{32}P]-phosphorothioate oligonucleotides after intravenous injection are shown in Figure 3A. Plasma half-life was approximately five minutes, while half-life in urine was much greater (approximately 28 min). Urine and plasma samples collected at various times were analyzed by electrophoresis on 15% polyacrylamide/7 M urea gels (Fig. 3B). Disappearance of phosphorothioated oligonucleotides was rapid in plasma. In urine, however, phosphorothioated oligonucleotides were stable without evidence of degradation for up to one hour.

To localize oligodeoxynucleotides in specific sites within kidney, renal tissue sections were examined by autoradiography and dark field microscopy (Fig. 4). After 15 minutes, oligonucleotide could be found in both proximal and distal tubules (Fig. 4B). After 30 minutes, the majority of counts were located within cells (Fig. 4 C, D). Abundant counts were present in Bowman's space and within glomerular epithelial cells (Fig. 4C). At 30 minutes, radioactivity appeared to be intracellular and also in aggregations along the brush border membrane (Fig. 4 C, D).

To explore the DNA binding activity from renal brush border proteins, renal cortical membrane preparations were affinity purified on biotin-labeled phosphorothioate oligonucleotides complexed to avidin agarose. Oligonucleotide binding proteins were

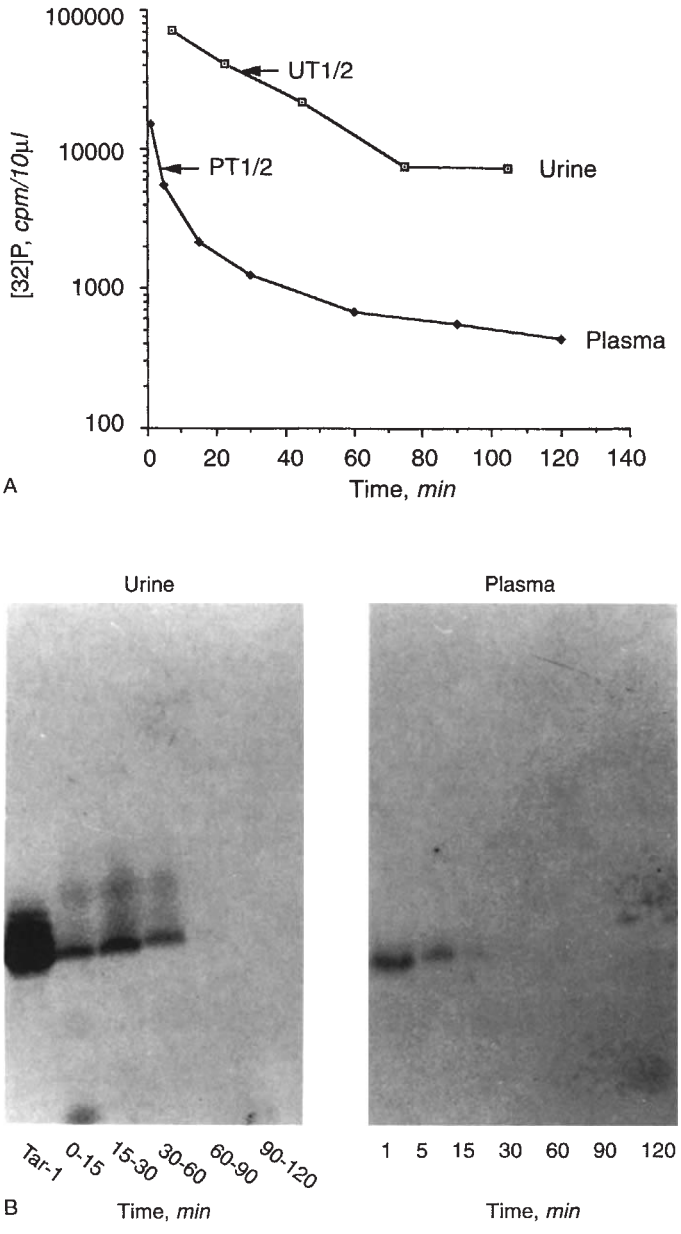

Fig. 3. *Time course of antisense oligonucleotide appearance in plasma (◆) and urine (⊡).* **(A)** Plasma and urine decay curves. The half life of oligonucleotide was 5 minutes in plasma but 28 minutes in urine. **(B)** Stability of phosphorothioate oligonucleotides in plasma and urine. Labeled oligonucleotide was detectable for only 5 minutes in plasma but could be detected intact in urine for up to 60 minutes. Methods: Plasma and urine were collected during *in vivo* clearance studies at the indicated intervals. [^{32}P] in samples was measured by liquid scintillation counter. At various times following the administration of labeled phosphorothioate oligonucleotides, plasma and urine were collected, mixed with an equal volume of loading buffer containing 80% formamide, and loaded on 15% acrylamide/7 M urea gel. The gel was exposed to X-ray film overnight. Mobility in electrophoresis is shown relative to a labeled Tar-1 oligonucleotide (20 mer) as a marker.

eluted in high salt buffer, concentrated by pressure filtration, and analyzed for DNA binding activity. As shown in Figure 5A, gel mobility shift assay of purified fractions demonstrated oligonucleotide binding activity in preparations from oligonucleotide matrix (lane 2) but absent from preparations from matrix alone (lane 5). To exclude the sequence dependence of binding, electrophoretic mobility shift assays (EMSA) were performed using proteins purified using the sequence of the HIV-1 transactivator response region (TAR) and competition was performed with either excess unlabeled TAR or excess oligonucleotides corresponding to the transcription factor SP1. Both TAR and SP1 sequences competed effectively (Fig. 5A, lanes 3 and 4, respectively), suggesting that binding was sequence independent.

Photochemical cross linking of nucleic acids to proteins has been a useful approach to identify unknown nucleic acid binding proteins and to characterize known protein:nucleic acid interactions. The cross linking of nucleic acids to proteins generates a covalent linkage between pyrimidine bases (that is, cytosine, thymine, or uracil) and certain amino acids [49]. In order to identify proteins which interact with phosphorothioate oligonucleotides, affinity-purified oligonucleotide binding proteins were UV irradiated in the presence of ^{32}P-labeled oligonucleotide. Analysis of polypeptides by polyacrylamide gel electrophoresis revealed specific oligonucleotide binding proteins of 46 kDa and 97 kDa (Fig. 5B). The 97 kDa polypeptide comigrated with the bovine serum albumin prestained marker. Since BSA (unstained) is faster migrating (66,600), this protein is probably not albumin. We cannot exclude the possibility that the 97 kDa is a dimer of the 46 kDa polypeptide. The 97 kDa band is barely detectable with protein preparations purified with biotin-agarose (no oligonucleotide control) (lane 3), however, there is a dramatic increase when proteins were affinity purified using an oligonucleotide containing matrix (lane 2), suggesting specificity. Identical results were observed in EMSA and UV cross linking assays when protein purification was accomplished using SP1 oligonucleotide (data not shown). These data provide additional evidence that the DNA-protein binding activity in renal brush border membrane is not dependent on the oligonucleotide sequence.

Discussion

The systemic delivery of chemically modified antisense oligonucleotides holds great promise for molecular therapy for many diseases including cancer, AIDS, and renal disease. Despite the great potential for clinical application, the molecular mechanism by which antisense molecules are taken up by cells has been remarkably difficult to determine. Previous studies that have addressed the half life and distribution of antisense *in vivo* have established that circulating oligonucleotides are degraded predominately in liver and that the kidney participates significantly in oligonucleotide excretion [41–43, 48]. In the present study, we find similar pharmacological handling of phosphorothioate antisense using sequences directed to potential targets for the treatment of HIV-1 infection. In addition, we find that oligonucleotides are cleared at a fraction of inulin clearance (30%) and that competition with unlabeled oligos increases the fractional excretion to 50% suggesting binding to either a plasma protein or to the renal brush border. Furthermore, we have found that renal brush border membrane exhibits significant DNA binding activity and we have isolated the majority of this activity to 46 kDa and 97 kDa proteins. Finally, this binding activity appears to be sequence-independent.

Previous studies have established the *in vivo* pharmacokinetics of systemically administered oligonucleotides [41–43, 48]. In the

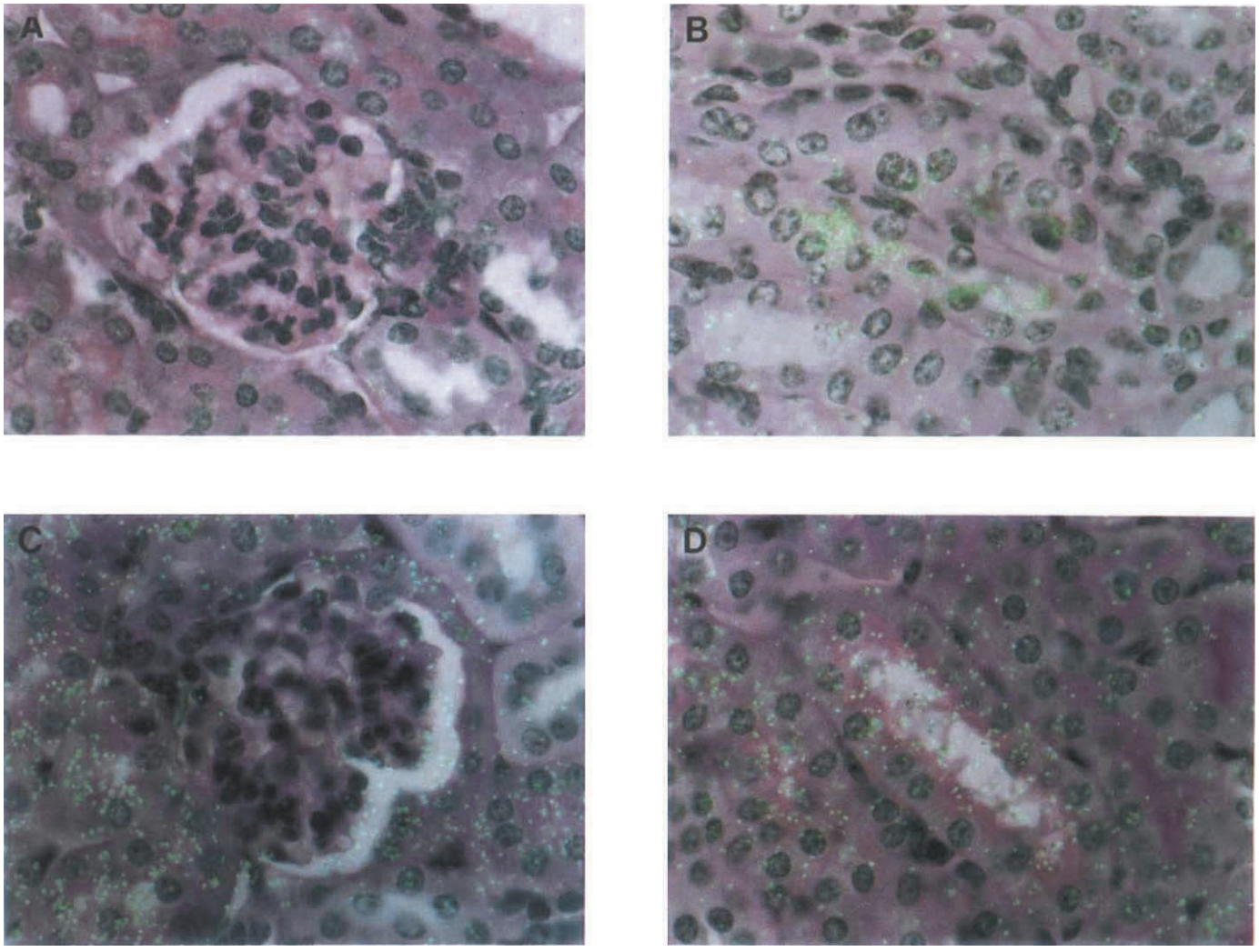

Fig. 4. *Autoradiography and dark field microscopy of labeled phosphorothioated antisense oligonucleotides in kidney.* Animals were injected intravenously with internally labeled [^{35}S] TAR oligonucleotides as described in **Methods**. Kidneys were harvested without injection (**A**), at 15 minutes (**B**), and 30 minutes (**C** and **D**) post-injection. Photomicrographs were made by superimposing bright field images and filtered dark field images of the same field of view to allow visualization of renal anatomy as well as the location of radiolabeled probe. Without antisense infusion background was minimal (A).

present study, our pharmacokinetic data were quite similar to previous investigators with the half-life of excretion of intact oligonucleotide in urine substantially longer than that in plasma. Significant binding to a circulating cell (platelet or leukocyte) or plasma proteins could account for this pattern of excretion with filtration of the oligonucleotide dependent upon the kinetics of dissociation of the S-deoxynucleotides from its binding site. Although antisense has been reported to be absorbed by platelets [50] and leukocytes [51], this possibility appears unlikely, since <5% of counts could be attributed to cellular binding *in vitro*. Furthermore, autoradiographic analysis of kidney sections after labeled antisense administration revealed the majority of label in the urinary space and renal tissue rather than on cells within capillaries. In this study, oligonucleotides appeared in abundance within Bowman's capsule, suggesting that they had gained entry to the urinary space by filtration. Because the molecular weight of a 20-mer is quite small (<7000 kDa), a size barrier to filtration appears to be unlikely. Previous studies have clearly demonstrate binding of phosphorothioate oligonucleotides to plasma proteins [52]. It is not clear how binding to plasma proteins affects the clearance results, but the rapid clearance of label from the plasma suggests that clearance is not greatly affected by this binding. Since unlabeled competition increased the fractional excretion of antisense, it appears more likely that the displacement of binding occurred within the renal urinary space during the process of reabsorption, rather than the displacement of oligonucleotides from plasma proteins. Combined with the autoradiographic evidence and gel mobility shift assay, these data support the hypothesis that specific binding to renal epithelium occurs.

Since specific binding of DNA clearly antedated the evolutionary exposure to systemic antisense administration, it is important to consider the identity of the endogenous substrate. Given the role of renal epithelial cells in antigen presentation, the uptake of small nucleic acids by a specific binding protein may have functional relevance in immune surveillance mechanisms for circulating DNA. In that regard, we have identified putative cell surface

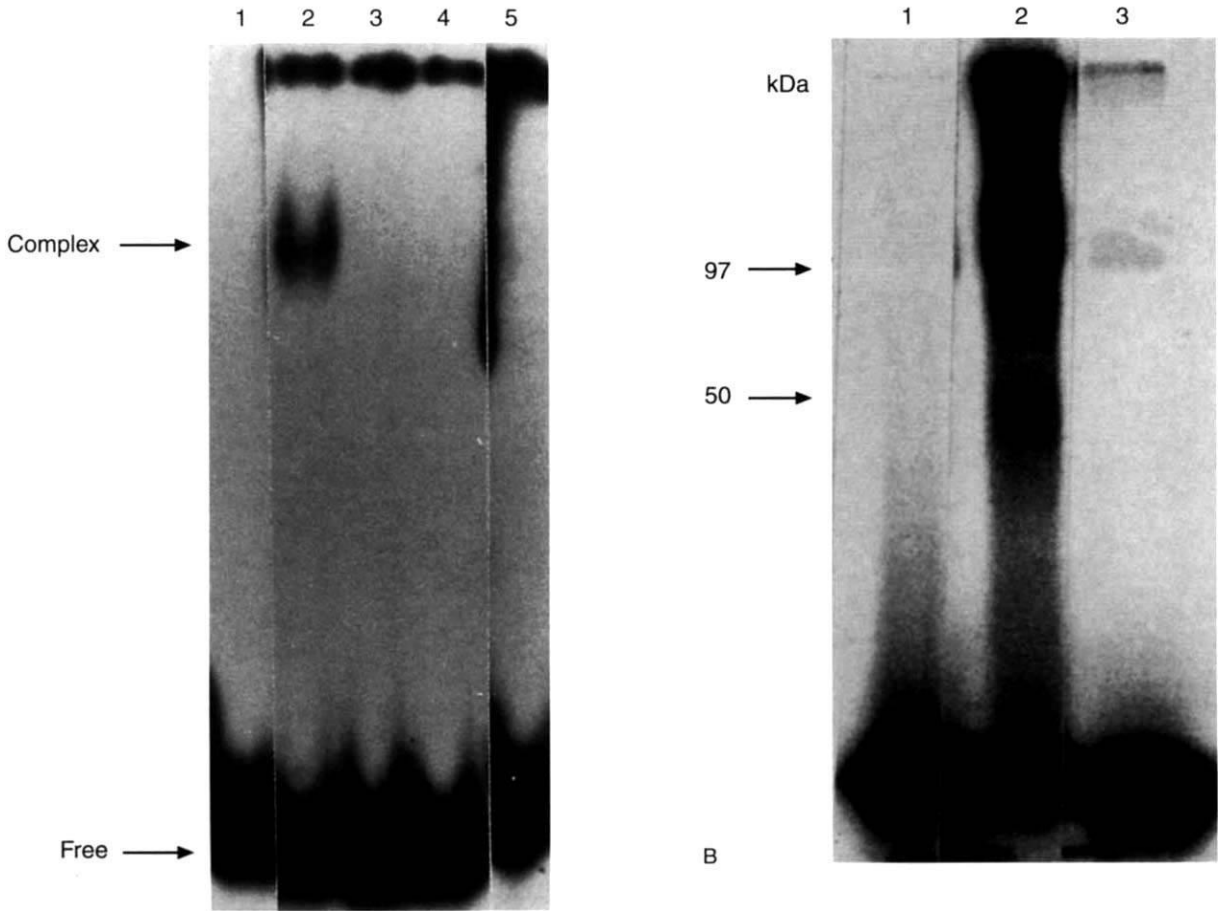

Fig. 5. *Electrophoretic mobility shift assay and UV cross linking of purified brush border membrane proteins.* Purified protein samples were incubated with [^{32}P] labeled DNA (100,000 cpm) in a buffer consisting of 400 mM NaCl, 10% glycerol, and 170 μg/ml poly dIdC for 20 minutes at room temperature. **(A)** EMSA of TAR-affinity purified brush border membrane oligonucleotide binding protein. Lane 1, free TAR oligonucleotide probe alone; lane 2, TAR oligonucleotide probe plus affinity purified protein; lane 3, identical to lane 2 with excess unlabeled TAR oligonucleotide; lane 4, identical to lane 2 with excess unlabeled SP1 oligonucleotide; lane 5, control protein incubated with labeled TAR oligonucleotide. **(B)** SDS-PAGE of UV crosslinked protein-oligonucleotide complexes. UV cross linking was performed as described in **Methods**. Lane 1, TAR oligonucleotide probe alone; lane 2, TAR affinity purified protein incubated with TAR oligonucleotide probe; lane 3, protein affinity purified using biotin-agarose resin (no oligonucleotide) incubated with labeled TAR oligonucleotide.

oligonucleotide receptor proteins of 46 kDa and 97 kDa from enriched preparations of purified brush border membranes.

Nucleic acid receptors have previously been proposed for various cell types [30, 51, 53–57] and the mechanisms of uptake in different cells may be dependent upon the presence and abundance of particular cell type-specific nucleic acid binding proteins. Given the preferential uptake of oligonucleotides in kidney and liver, these organs appear to be rich in specific receptors for DNA. Although antisense uptake has been studied in liver [33], there has been little investigation of oligonucleotide metabolism in the kidney. Based on the molecular size estimated by UV cross-linking studies, the polypeptides we have identified in affinity purified preparations from brush border membranes appear to be different from those previously described using other cell types. These differences may reflect mechanistic diversity both functionally and in DNA metabolism. Since the kidney exhibits preferential oligonucleotide uptake, it appears likely the renal proximal tubule cells have either cell type-specific nucleic acid receptors or are specialized in their abundance of a more general receptor for DNA. Future studies will determine if the polypeptide that we have identified is specific for or enriched in renal epithelial cells.

The preferential localization and uptake of oligonucleotides in kidney emphasizes the potential utility of antisense therapy in the treatment of renal diseases, particularly in tubular and interstitial disease states. These data also suggest that the kidney may be a site of toxicity for antisense therapy for other diseases. Recently, nephrotoxicity was observed with high dose *in vivo* antisense administration [58]. Further elucidation of the mechanisms of renal uptake of antisense oligonucleotides will enable rational strategies for chemical modifications to enhance renal targeting and minimize nephrotoxicity. These considerations may be relevant in efforts to target antisense to other organs as well, where the diversion of antisense away from the kidney would be desirable to minimize toxicity and to increase the bioavailability at

other sites. The definition of specific mechanisms for DNA uptake by renal cells remains unclear, but is necessary for continued progress in the use of systemic antisense for molecular therapy.

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References

1. Mirabelli CK, Bennett CF, Anderson K, Crooke ST: In vitro and in vivo pharmacologic activities of antisense oligonucleotides. *Anticancer Drug Des* 6:647–661, 1991
2. Crooke ST: Progress toward oligonucleotide therapeutics: Pharmacodynamic properties. *FASEB J* 7:533–539, 1993
3. Boiziau C, Kurfurst R, Cazenave C, Roig V, Thuong NT, Toulme JJ: Inhibition of translation initiation by antisense oligonucleotides via an RNase-H independent mechanism. *Nucl Acids Res* 19:1113–1119, 1991
4. Baker BF, Miraglia L, Hagedorn CH: Modulation of eucaryotic initiation factor-4E binding to 5′-capped oligoribonucleotides by modified anti-sense oligonucleotides. *J Biol Chem* 267:11495–11499, 1992
5. Stein CA, Cheng YC: Antisense oligonucleotides as therapeutic agents—Is the bullet really magical? *Science* 261:1004–1012, 1993
6. Kawasaki AM, Martin JF, Guinosso CJ, Zounes MC, Springer RS, Hoke GD, Freier SM, Cook PD: Synthesis and biophysical studies of 2′-d-ribo-f modified oligonucleotides. (abstract) *Int Union of Biochemistry Conf on Nucleic Acid Therapeutics* 1991, p 71
7. Vlasov VV, Iurchenko LV: [Mechanism for suppression mRNA translation with antisense oligonucleotides]. *Mol Biol* (Moskow) 24: 1157–1161, 1990 (in Russian)
8. Li G, Lisziewicz J, Sun D, Zon G, Daefler S, Wong-Staal F, Gallo RC, Klotman ME: Inhibition of Rev activity and human immunodeficiency virus type 1 replication by antisense oligodeoxynucleotide phosphorothioate analogs directed against the Rev-responsive element. *J Virol* 67:6882–6888, 1993
9. Goodchild J, Agrawal S, Civeira MP, Sarin PS, Sun D, Zamecnik PC: Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* 85:5507–5511, 1988
10. Agrawal S, Tang JY: GEM 91—An antisense oligonucleotide phosphorothioate as a therapeutic agent for AIDS. (editorial) *Antisense Res Dev* 2:261–266, 1992
11. Jaskulski D, Deriel JK, Mercer WE, Calabretta B, Baserga R: Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science* 240:1544–1546, 1988
12. Maier JA, Voulalas P, Roeder D, Maciag T: Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. *Science* 249:1570–1574, 1990
13. Vickers T, Baker BF, Cook PD, Zounes M, Buckheit RW Jr, Germany J, Ecker DJ: Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element. *Nucl Acids Res* 19: 3359–3368, 1991
14. Doerre S, Sista P, Sun SC, Ballard DW, Greene WC: The c-rel protooncogene product represses NF-kappa B p65-mediated transcriptional activation of the long terminal repeat of type 1 human immunodeficiency virus. *Proc Natl Acad Sci USA* 90:1023–1027, 1993
15. Cowsert LM, Fox MC, Zon G, Mirabelli CK: In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: Potential treatment for genital warts. *Antimicrob Agents Chemother* 37:171–177, 1993
16. Bielinska A, Shivdasani RA, Zhang LQ, Nabel GJ: Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science* 250:997–1000, 1990
17. Potts JD, Dagle JM, Walder JA, Weeks DL, Runyan RB: Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor beta 3. *Proc Natl Acad Sci USA* 88:1516–1520, 1991
18. Ratakczal MZ, Kant JA, Luger SM, Hijiya N, Zhang J, Zon G, Gewirtz AM: In vivo treatment of human leukemia in a scid mouse model with c-myb antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* 89:11823–11827, 1992
19. Kitajima I, Shinohara T, Bilakovics J, Brown DA, Xu X, Nerenberg M: Ablation of transplanted HTLV-I Tax-transformed tumors in mice by antisense inhibition of NF-kappa B. *Science* 258:1792–1795, 1992
20. Le Doan T, Chavany C, Helene C: Antisense oligonucleotides as potential antiviral and anticancer agents. *Bull Cancer* (Paris) 76:849–852, 1989
21. Tidd DM: Synthetic oligonucleotides as therapeutic agents. (editorial) *Br J Cancer* 63:6–8, 1991
22. Bayever E, Iversen P, Smith L, Spinolo J, Zon G: Systemic human antisense therapy begins. (editorial) *Antisense Res Dev* 2:109–110, 1992
23. Calabretta B, Skorski T, Szczylik C, Zon G: Prospects for gene-directed therapy with antisense oligodeoxynucleotides. *Cancer Treat Rev* 19:169–179, 1993
24. Zon G, Geiser TG: Phosphorothioate oligonucleotides: Chemistry, purification, analysis, scale-up and future directions. *Anticancer Drug Des* 6:539–568, 1991
25. Lesnik EA, Guinosso CJ, Kawasaki AM, Sasmor H, Zounes M, Cummins LL, Ecker DJ, Cook PD, Freier SM: Oligodeoxynucleotides containing 2′-O-modified adenosine: Synthesis and effects on stability of DNA:RNA duplexes. *Biochemistry* 32:7832–7838, 1993
26. Kibler-Herzog L, Zon G, Uznanski B, Whittier G, Wilson WD: Duplex stabilities of phosphorothioate, methylphosphonate, and RNA analogs of two DNA 14-mers. *Nucl Acids Res* 19:2979–2986, 1991
27. Tang JY, Temsamani J, Agrawal S: Self-stabilized antisense oligodeoxynucleotide phosphorothioates: Properties and anti-HIV activity. *Nucl Acids Res* 21:2729–2735, 1993
28. Rothenberg M, Johnson G, Laughlin C, Green I, Cradock J, Sarver N, Cohen JS: Oligodeoxynucleotides as anti-sense inhibitors of gene expression: Therapeutic implications. *J Natl Cancer Inst* 81:1539–1544, 1989
29. Zhao Q, Matson S, Herrera CJ, Fisher E, Yu H, Krieg AM: Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res Dev* 3:53–66, 1993
30. Geselowitz DA, Neckers LM: Analysis of oligonucleotide binding, internalization, and intracellular trafficking utilizing a novel radiolabeled crosslinker. *Antisense Res Dev* 2:17–25, 1992
31. Loke SL, Stein CA, Zhang XH, Mori K, Nakanishi M, Subasinghe C, Cohen JS, Neckers LM: Characterization of oligonucleotide transport into living cells. *Proc Natl Acad Sci USA* 86:3474–3478, 1989
32. Yakubov L, Yurchenko L, Nechaeva M, Rykova E, Karaymshev V, Tonkinson J, Vlassov V, Stein CA: Interaction of oligonucleotides with cellular receptors. (abstract) *Nucl Acids Symp Ser* 1991 p 311
33. Vlasov VV, Ivanova EM, Nechaeva MV, Rykova EI, Iakubov LA, Zon D: [Affinity modification of proteins, binding nucleic acids in mammalian cells, by alkylating derivatives of oligonucleotides]. *Biokhimiia* 58:962–966, 1993 (in Russian)
34. Chin DJ, Green GA, Zon G, Szoka FC Jr, Straubinger RM: Rapid nuclear accumulation of injected oligodeoxyribonucleotides. *New Biol* 2:1091–1100, 1990
35. Leonetti JP, Mechti N, Degols G, Gagnor C, Lebleu B: Intracellular distribution of microinjected antisense oligonucleotides. *Proc Natl Acad Sci USA* 88:2702–2706, 1991
36. Mechti N, Leonetti JP, Clarenc JP, Degols G, Lebleu B: Nuclear location of synthetic oligonucleotides microinjected somatic cells: Its

implication in an antisense strategy. *Nucl Acids Symp Ser* 147–150, 1991
37. Noonberg SB, Garovoy MR, Hunt CA: Characteristics of oligonucleotide uptake in human keratinocyte cultures. *J Invest Dermatol* 101:727–731, 1993
38. Yakubov LA, Deeva EA, Zarytova VF, Ivanova EM, Ryte AS, Yurchenko LV, Vlassov VV: Mechanism of oligonucleotide uptake by cells: Involvement of specific receptors? *Proc Natl Acad Sci USA* 86:6454–6458, 1989
39. Hefeneider SH, Bennett RM, Pham TQ, Cornell K, Mccoy SL, Heinrich MC: Identification of a cell-surface DNA receptor and its association with systemic lupus erythematosus. *J Invest Dermatol* 94:79S–84S, 1990
40. Hefeneider SH, McCoy SL, Morton JI, Bakke AC, Cornell KA, Brown LE, Bennett RM: DNA binding to mouse cells is mediated by cell-surface molecules: The role of these DNA-binding molecules as target antigens in murine lupus. *Lupus* 1:167–173, 1992
41. Agrawal S, Temsamani J, Tang JY: Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc Natl Acad Sci USA* 88:7595–7599, 1991
42. Goodarzi G, Watabe M, Watabe K: Organ distribution and stability of phosphorothioated oligodeoxyribonucleotides in mice. *Biopharm Drug Dispos* 13:221–227, 1992
43. Cossum PA, Truong L, Owens SR, Markham PM, Shea JP, Crooke ST: Pharmacokinetics of a ^{14}C-labeled phosphorothioate oligonucleotide, ISIS 2105, after intradermal administration to rats. *J Pharmacol Exp Ther* 267:1181–1190, 1993
44. Mannon RB, Sundar SK, Sanfilippo FP, Coffman TM: Alterations in renal interleukin-1 production during kidney transplant rejection in the rat. The effects of high-dose methylprednisolone. *Transplantation* 56:1157–1162, 1993
45. Stein A, Iversen PL, Subasinghe C, Cohen JS, Stec WJ, Zon G: Preparation of ^{35}S-labeled polyphosphorothioate oligodeoxyribonucleotides by use of hydrogen phosphonate chemistry. *Anal Biochem* 188:11–16, 1990
46. Evers C, Haase W, Murer H, Kinne R: Properties of brush border vesicles isolated from rat kidney cortex by calcium precipitation. *Membr Biochem* 1:203–219, 1978
47. Bruggeman LA, Burbelo PD, Yamada Y, Klotman PE: A novel sequence in the type IV collagen promoter binds nuclear proteins from Engelbreth-Holm-Swarm tumor. *Oncogene* 7:1497–1502, 1992
48. Goodchild J, Kim B, Zamecnik PC: The clearance and degradation of oligodeoxynucleotides following intravenous injection into rabbits. *Antisense Res Dev* 1:153–160, 1991
49. Smith KC, Meun DHC: Kinetics of the photochemical addition of ^{35}S cysteine to polynucleotides and nucleic acids. *Biochemistry* 7:1033–1037, 1968
50. Clejan L, Menahem H: Binding of deoxyribonucleic acid to the surface of human platelets. *Acta Haematol* 58:84–88, 1977
51. Bennett RM, Gabor GT, Merritt MM: DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. *J Clin Invest* 76:2182–2190, 1985
52. Bigelow JC, Chrin LR, Mathews LA, McCormick JJ: High performance liquid chromatographic analysis of phosphorothioate analogues of oligodeoxynucleotides in biological fluids. *J Chromatogr* 533:133–140, 1990
53. Kitajima I, Shinohara T, Minor T, Bibbs L, Bilakovics J, Nerenberg M: Human T-cell leukemia virus type I tax transformation is associated with increased uptake of oligodeoxynucleotides in vitro and in vivo. *J Biol Chem* 267:25881–25888, 1992
54. Iversen PL, Crouse D, Zon G, Perry G: Binding of antisense phosphorothioate oligonucleotides to murine lymphocytes is lineage specific and inducible. *Antisense Res Dev* 2:223–233, 1992
55. Ohkubo H, Kageyama R, Ujihara M, Hirose T, Inayama S, Nakanichi S: Cloning and sequence analysis of cDNA for rat angiotensinogen. *Proc Natl Acad Sci USA* 80:2196–2200, 1990
56. Gill PS, Rarick M, McCutchan JA, Slater L, Parker B, Muchmore E, Bernstein Singer M, Akil B, Espina BM, Krailo M, et al: Systemic treatment of AIDS-related Kaposi's sarcoma: Results of a randomized trial. *Am J Med* 90:427–433, 1991
57. Goodarzi G, Watabe M, Watabe K: Binding of oligonucleotides to cell membranes at acidic pH. *Biochem Biophys Res Commun* 181: 1343–1351, 1991
58. Sarmiento UM, Perez JR, Becker JM, Narayanan R: In vivo toxicological effects of rel A antisense phosphorothioates in CD-1 mice. *Antisense Res Dev* (in press)