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Enhanced resistance to nuclease degradation of nucleic acids complexed to asialoglycoprotein-polylysine carriers

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ABSTRACT

We have previously shown targeting of DNA to hepatocytes using an asialoorosomucoid-polylysine (AsOR-PL) carrier system. The AsOR-PL conjugate condenses DNA and facilitates entry via specific receptor-ligand interactions. In these studies, our objective was to determine if AsOR-PL conjugates protect bound DNA from nuclease attack. Double-stranded plasmid or single-stranded oligonucleotide DNA, alone or bound to conjugate, was incubated under conditions mimicking those encountered during *in vitro* and *in vivo* transfections. The results showed that complexed DNA was effectively protected from degradation by serum nucleases. Degradation of single-stranded oligonucleotides was inhibited 3- to 6-fold in serum during 5 hours of incubation. For complexed plasmids, greater than 90% remained full-length during 1.5 and 3 hour incubations in serum or culture medium containing 10% serum, respectively. Uncomplexed plasmid was completely degraded after 15 minutes in serum or 60 minutes in medium. In cell lysates, the conjugate was not effective in inhibiting endonuclease activity; plasmids were readily converted from supercoiled to open circular and linear forms. However, the resultant nicked forms were substantially protected from further degradation during one hour of incubation compared to plasmid alone. Under all conditions complexed DNA did not readily dissociate from the conjugate. Overall, for both single and double-stranded DNA, AsOR-PL conjugates conferred substantial protection from nuclease degradation.

INTRODUCTION

We have previously described a DNA carrier system capable of targeting DNA specifically to hepatocytes based on the presence of receptors on these cells that recognize galactose-terminal (asialo-) glycoproteins. Binding of DNA complexes by asialoglycoprotein receptors results in internalization within membrane limited vesicles. These and other ligand-based DNA carrier systems have been shown to deliver foreign DNA to result in new gene expression *in vitro* (1, 2) and *in vivo* (3). In these studies, the genes were delivered in the form of double-stranded bacterial plasmids. However, recently, the system was also used to deliver single-stranded DNA in the form of antisense oligonucleotides to inhibit endogenous gene expression (4) The conditions to which the DNAs were exposed in these experiments were hostile. Substantial nuclease activities have been shown to be present in serum and some intracellular compartments, e.g., lysosomes. Indeed, previous investigators have demonstrated that naked DNA exposed to cell lysates and serum nucleases are readily degraded (5–7). The objective of the current experiments was to assess the stability of DNA held in a complex with AsOR-PL conjugates in various environments that mimicked a variety of transfection conditions. Incubations were performed in culture medium containing fetal bovine serum to simulate extracellular conditions of *in vitro* transfection, in fresh whole rat serum to simulate transport through the circulation in *in vivo* transfections, and in a crude cell lysate preparation to simulate an intracellular environment following internalization of DNA.

MATERIALS AND METHODS

DNA species

To assess carrier-mediated protection of nucleic acids, two types of DNA were prepared: 1) double stranded DNA in the form of a 5.2 kbp plasmid, pSV-HBV$_2$, and 2) a single stranded 21-mer oligodeoxyribonucleic acid synthesized with phosphodiester linkages using an Applied Biosystems DNA synthesizer, or supplied as an HPLC purified product by the Midland Certified Reagent Co. (Midland, TX). Plasmid DNA was isolated from DH5α *E.coli* by standard alkaline lysis procedures (8), and purified by double banding in CsCl gradients. For quantitative studies, the oligonucleotide was 5′-end labeled with ^{32}P using T4-polynucleotide kinase in the presence of adenosine 5′ (^{32}P) triphosphate (3000 Ci/mmol, Amersham Corp., Arlington Heights, IL). The labeled oligomer was isolated by

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chromatography through a P2 Bio-Gel column (Bio-Rad, Richmond, CA).

Targetable DNA carrier

Orosomucoid was prepared from pooled human plasma (9). Terminal sialic acid residues were removed by mild acid hydrolysis (10) to form asialoorosomucoid. A conjugate between asialoorosomucoid (AsOR) and poly-L-lysine (PL) (21 kD or 41 kD mean molecular weight; Sigma Chemical Co., St Louis, MO) was prepared at pH 7.4 as described (4, 11) with starting components in a molar ratio of 1:1. The coupling agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Chemical Co., Rockford, IL), was added in a 154-fold molar excess over poly-L-lysine. After 24 hours, the conjugate product was purified by preparative gel electrophoresis using an acid-urea polyacrylamide gel system. Bands were eluted with 0.35 M β-alanine-acetic acid, pH 4.8, and checked for UV absorption at 230 nm. The second peak eluted was identified and confirmed as conjugate purified to homogeneity by analytical acid-urea gel electrophoresis (12). The purified conjugate was dialyzed against 0.025 M Tris, 0.001 M EDTA through membranes with 12–14 kD exclusion limits.

AsOR-PL–DNA complexes

Complexes composed of DNA bound to AsOR-PL conjugates were prepared by slow addition of AsOR-PL in 0.15 M NaCl to a specific amount of plasmid or oligonucleotide DNA dissolved in either H_2O or 10 mM Tris, pH 7.5, 1 mM EDTA solution (TE). The optimal ratio of AsOR-PL conjugate to DNA desired in the final complex was determined by retardation assays in agarose gels as described previously (1). The ratios of the complexes used in this study, on a weight to weight basis, were 2:1 for plasmid DNA, and 1.9:1 for the oligomer, respectively.

Incubation conditions to assess DNA stability

To evaluate the ability of AsOR-PL conjugates to protect nucleic acids from degradation, DNA, either free or complexed to AsOR-PL, were incubated at 37°C for various times in saline solution (0.15M NaCl in H_2O), tissue culture medium containing fetal bovine serum (FBS, Life Technologies, Gaithersburg, MD), fresh Sprague-Dawley rat serum obtained from whole blood by retroorbital puncture, or a crude cell lysate preparation. The cell lysate was obtained from approximately $2-8\times10^6$ HepG2 or NIH 3T3 cells that were washed in phosphate buffered saline (PBS), pelleted, and completely disrupted by 6 cycles of freeze-thawing in 0.25 M Tris, pH 7.4. Supernatant from the broken-cell suspension was stored at −70°C prior to use. Immediately before use the lysate was treated for 90 minutes at 37°C with 1.7 units of RNase (Life Technologies) and 3 units of DNase I (Boehringer-Mannheim, Indianapolis, IN) per microliter of supernatant to eliminate competition for conjugate binding by endogenous nucleic acids.

For single-stranded ^{32}P-labeled oligonucleotide, 1 μg of DNA was incubated with an equal volume of the various media described above. The tissue culture medium consisted of 2× Modified Eagle's Medium (MEM, Life Technologies) containing 20% FBS. After incubation of the oligomers, samples were frozen in liquid N_2, and stored at −70°C for subsequent dissociation of the DNA and analysis.

For plasmids, 3 μg of DNA in 10 μl total volume was added to either 180 μl of 1×MEM containing 10% FBS, 120 μl of serum, or 10 μl of crude cell lysate. After incubation the samples were processed immediately to dissociate the DNA from the protein conjugate, as described below.

Dissociation of DNA from AsOR-PL complexes

Because nucleic acids bind to AsOR-PL conjugates by non-specific electrostatic interactions, degraded DNA fragments could remain complexed with AsOR-PL. To examine the actual physical state of the DNA, it was necessary to dissociate it from the protein conjugate following incubation. For studies using oligonucleotides, half of each sample was treated with 50 units of heparin (Life Technologies) per microgram of DNA for 5 minutes at 25°C to free the oligomer from conjugate by competitive displacement.

For plasmid complexes, nucleases were heat inactivated at 65°C for 20 minutes, cooled slowly to room temperature, then the samples were dissociated by digestion of the polylysine with an equal volume of 10× trypsin (Life Technologies) at 37°C for 30 minutes. The mixture was extracted with phenol and DNA was precipitated with ethanol. The pelleted DNA was resuspended in TE buffer for analysis.

Analysis of DNA degradation

To assess oligonucleotide degradation, each sample, stored frozen, was rapidly thawed at 37°C, and half was dissociated from complex, as described above, before electrophoresis through 2% SeaPlaque GTG low melt agarose (FMC BioProducts, Rockland, ME) gels containing ethidium bromide. The remaining half was directly applied to the gels as native complex. After electrophoresis the radiolabel was directly visualized and quantitated in the gels using a Packard Instant*Imager* (Packard Instrument Co., Meriden, CT). Visualization was also accomplished by autoradiography on to XAR film (Eastman Kodak, Rochester, NY). Quantitation was confirmed by cutting each gel lane into 0.5 cm sections which where dissolved by heating to 75°C, mixed with scintillation fluid (National Diagnostics) and counted in a Beckman LS6000 liquid scintillation counter (Beckman Instruments, Palo Alto, CA). The radioactive bands in the gel that corresponded to undegraded free oligonucleotide and complexed DNA were identified by comparison to untreated standards. All radioactivity found to migrate faster than free DNA was considered to be degraded. The amount of intact and degraded oligomer remaining after incubation was calculated as percentages of the total radioactivity found within the sample lane. All analyses were performed in duplicate or triplicate and the results expressed as the mean ± S.E.

For analysis of plasmid degradation, the entire sample from each incubation was treated to dissociate the DNA from conjugate as described above, and electrophoresed through a 1% agarose gel. The DNA was transferred onto nylon membrane by capillary action and UV cross-linked to the membrane. The DNA was hybridized to a nick-translated fluorescein-labeled DNA probe complementary to the plasmid sequence and visualized by chemiluminescent autoradiography using the ECL detection system (Amersham). Autoradiographic bands representing (in order of migration from the top of the gel) open circular, linear, supercoiled, and degraded DNA were quantitated by densitometry using a Pharmacia 1D ImageMaster system (Pharmacia Biotech Inc., Piscataway, NJ). The relative amount of each DNA species remaining after incubation was calculated by dividing by the amount of that form found at time zero.

RESULTS

Inhibition of oligonucleotide DNA degradation by conjugate

For studies on single-stranded oligodeoxynucleotide degradation, a 21-mer with a conventional phosphodiester backbone was used.

It has been shown that this type of DNA is primarily degraded by 3′ exonucleases found in serum or medium containing serum (7). Labeling of the oligomer with ^{32}P was done at its 5′-end to increase the likelihood that residual degradation products retained the label. However, loss of radioactivity without actual cleavage of DNA could occur by dephosphorylation at the 5′-terminus with long exposure to natural environments such as serum or cell lysates. Thus, loss of radioactivity alone would not be a reliable marker for degradation of oligonucleotides. For this reason a combination of methods were used to detect

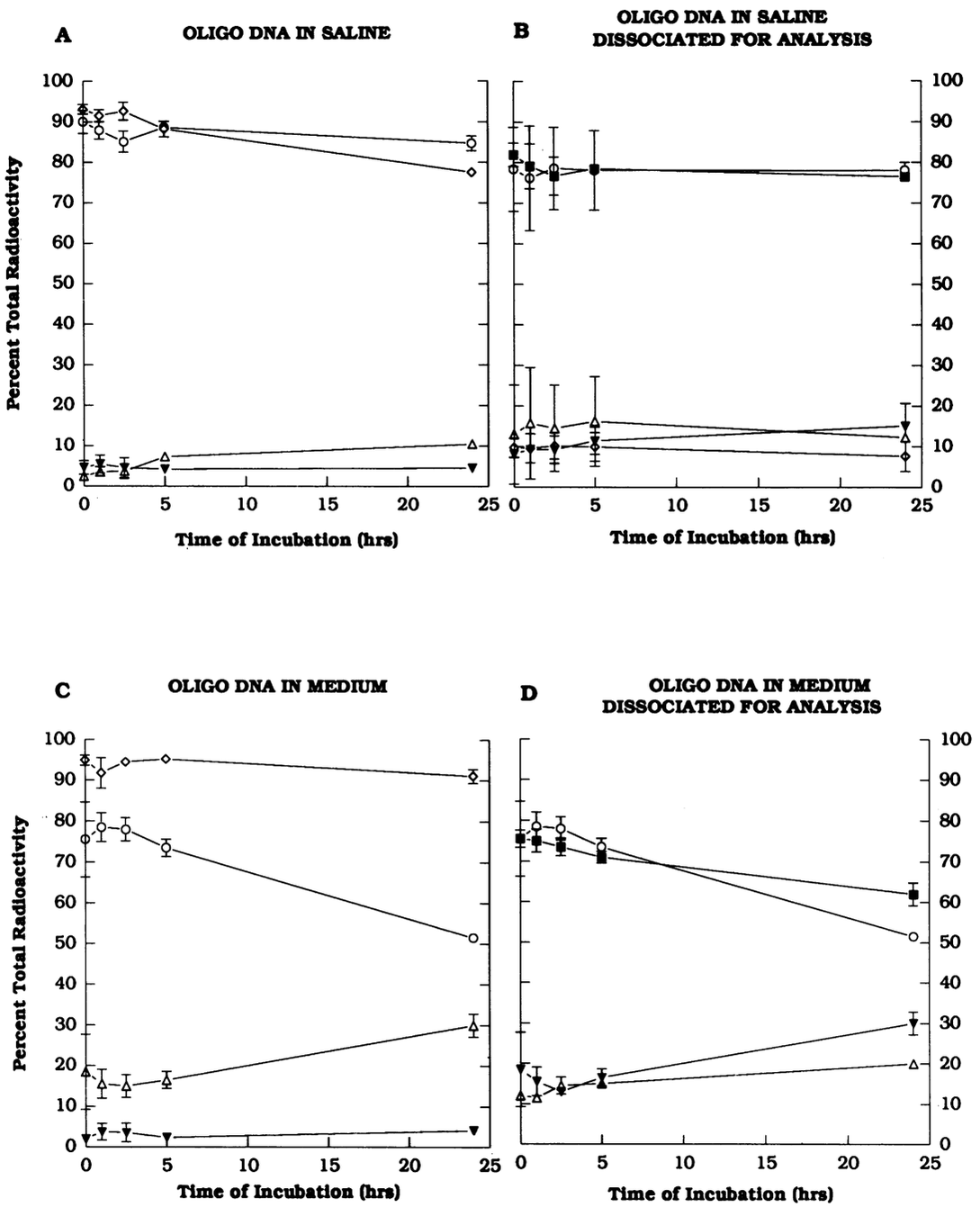

Figure 1. Quantitation of residual radioactivity, originally present as 5′-labeled 21-mer phosphodiester oligodeoxynucleotide, as a function of time after incubation. Results are expressed as percentage of total radioactivity measured in the lane. In panels **A** and **B** samples were incubated in saline solution, while samples in panels **C** and **D** were incubated in tissue culture medium containing FBS. In panels B and D samples were treated with heparin prior to electrophoresis to dissociate the DNA from conjugate, as described in Materials and Methods. Samples in panels A and C were not treated with heparin. Individual DNA species were identified by comparison to control DNA, either free or complexed. All radioactivity found to migrate faster than intact free DNA was considered to be degraded. After electrophoresis the radiolabel in each lane was directly measured using a Packard Instant*Imager* and visualized by autoradiography and ethidium bromide staining. The quantitation was confirmed by cutting each lane into sections for measurement by scintillation counting as described in Material and Methods. All analyses were performed in duplicate or triplicate and the results expressed as means ± S.E. Symbols are as follows: ○ Intact DNA from free DNA; Δ Degraded DNA from free DNA; ■ Intact DNA from complex; ▼ Degraded DNA from complex; ◇ Complexed DNA remaining as complex

degradation of the 21-mer after incubation: 1) electrophoretic migration in gels visualized by ethidium bromide staining, 2) direct measurement of radioactivity as a function of migration distance, and 3) autoradioagraphy. Figures 1 and 2 show the percentages of total radioactivity from each sample that was observed as either DNA still associated with complex, full-length oligonucleotide, or fast migrating degraded oligomer, after incubation in the four conditions used in these studies. The results depicted in panels A and C of both figures show the relative amount of DNA that remained complexed during incubation, while in panels B and D, DNA was dissociated from conjugate by heparin treatment after incubation in order to detect any degraded DNA that remained bound to conjugate. Figure 1, panel A shows that the amount of intact oligomer originally present as free DNA decreased only slightly (<5%) over 24 hours in saline solution. The graph also shows that complexed DNA in saline solution remained in the form of a complex with less than 10% dissociation through 24 hours. Treatment with heparin prior

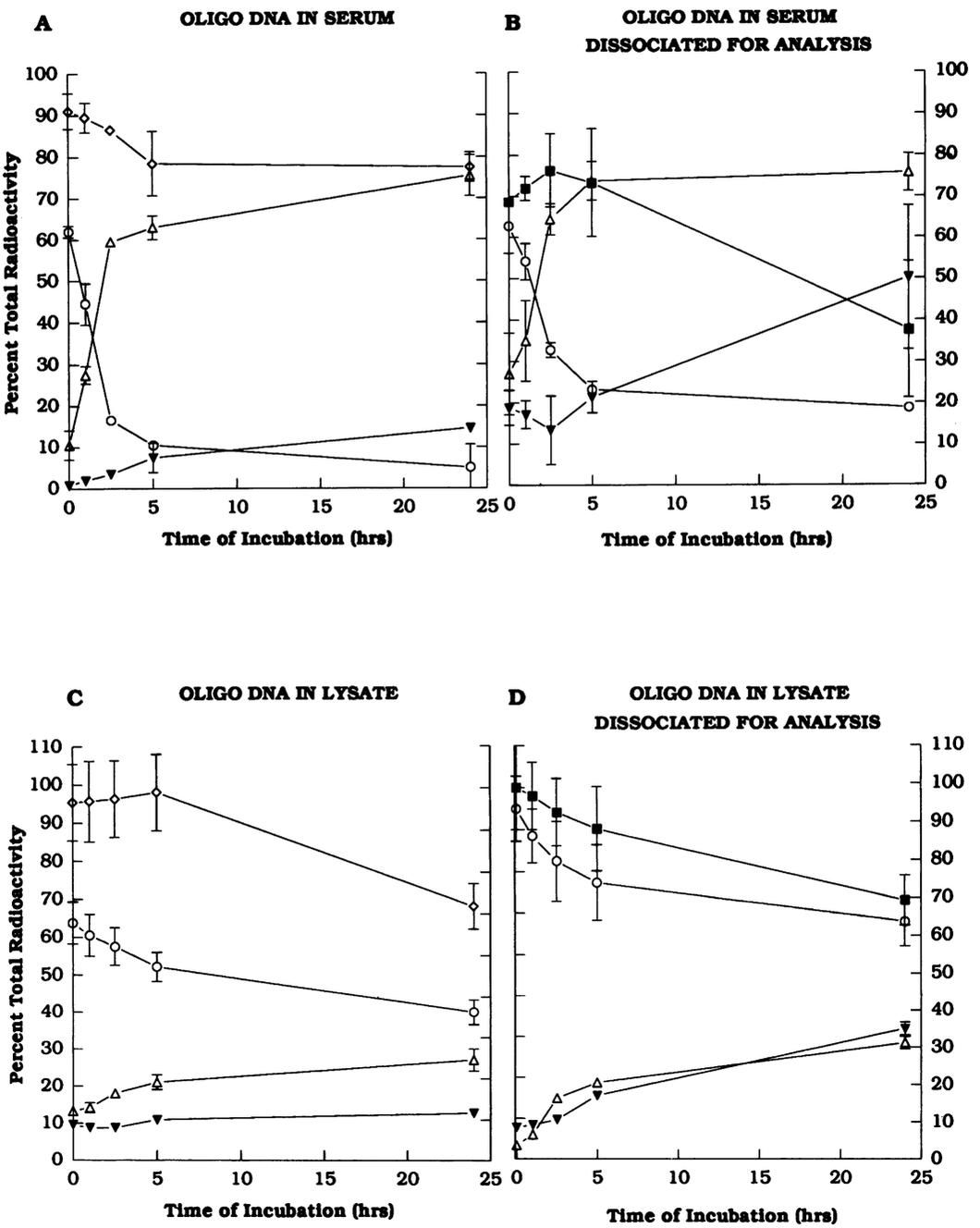

Figure 2. Quantitation of residual radioactivity, originally present as 5'-labeled oligodeoxynucleotide, after incubation in serum or cell lysate. Incubations in fresh rat serum are shown in panels **A** and **B**, incubations in cell lysate in panels **C** and **D**. Samples in panels B and D were first treated with heparin prior to electrophoresis. All analyses were performed in duplicate or triplicate and the results expressed as means ± S.E. The symbols used are the same as in Figure 1.

to analysis resulted in dissociation of more than 90% of complexed DNA to free DNA (Fig. 1B), and confirmed that little or no degradation of oligonucleotide occurred in saline solution. These results establish that AsOR-PL-DNA complexes are stable in the absence of nucleases. There is little or no spontaneous dissociation and DNA was not damaged nor degraded by complexation. Visualization of gels by ethidium bromide and autoradiography and quantitation by scintillation spectroscopy showed very similar results to these presented (data not shown).

Comparison of the zero time points in Figures 1 and 2, panels A and C versus panels B and D show that heparin-treatment prior to analysis had, at most, a very slight effect on degradation of complexed DNA and no effect on degradation of free oligomer. It is possible that during the brief time period between heparin displacement of DNA from complex and electrophoresis a small amount of degradation of newly freed oligomer took place. Thus the inhibition of degradation observed with complexed oligonucleotides may be an underestimation of the actual amount of protection afforded by AsOR-PL conjugates.

When free DNA was incubated in medium containing FBS, intact oligomer was observed to decrease by 25% in 24 hours and this decrease was accompanied by a 20% increase in degraded DNA (Fig. 1C and 1D). However, the amount of DNA retained in complex remained unchanged throughout the incubation (Fig. 1C). Dissociation (Fig. 1D) showed that about 5% of the DNA originally in complex was degraded after 5 hours and 10% after 24 hours. Again, the decrease was accompanied by a corresponding increase in degraded DNA. Visualization of the gels and quantitation by scintillation spectroscopy (data not shown) produced identical results, as was the case in all other experiments. The difference in the amount of degradation between free and complexed oligonucleotide was small and only apparent at 24 hours. This may be due to a relatively low level of nuclease activity present under our incubation conditions. It is possible that longer incubations under these conditions would have produced greater differences.

Incubation in fresh rat serum demonstrated much more dramatic differences in degradation between free and complexed oligonucleotide. The amount of free intact oligomer decreased 3- to 6-fold during the first 5 hours of incubation, from 60–65% of total radioactivity to 10–20% (Fig. 2A and B), accompanied by a corresponding increase in degraded DNA. After the same duration of incubation, complexed oligonucleotide decreased by only 10%, while heparin displacement showed no significant decrease in the amount of intact oligomer released from complex (Fig. 2B). Only after 24 hours of incubation in serum did complexed oligonucleotide sustain substantial degradation. Thus, binding of AsOR-PL conjugate to single-stranded oligonucleotide greatly slowed the rate at which the DNA was degraded in serum.

Representative electrophoretic gels of DNA incubated in serum and visualized by ethidium bromide staining and autoradiography are shown in Figures 3 and 4. Figure 3A and B shows that over a 24 hour incubation, the vast majority of complexed DNA remained at the top of the gel, indicating that the DNA remained bound to conjugate. Free DNA, lanes 1 to 5, was rapidly degraded. After 2.5 to 5 hours of incubation there is little intact oligomer remaining. Panel B shows that radiolabel appeared to be lost from free DNA to a similar extent as the loss of ethidium bromide staining.

Heparin treatment of free DNA prior to analysis had no significant effect on uncomplexed DNA. Figure 4A, lanes 1 to 5, is nearly identical to Figure 3A, lanes 1 to 5. Dissociation of complexed DNA that had been incubated in serum, lanes 6–10, revealed that the majority of the DNA in the complex remained intact through 5 hours of incubation. However, substantial degradation occurred by 24 hours (lane 10). The

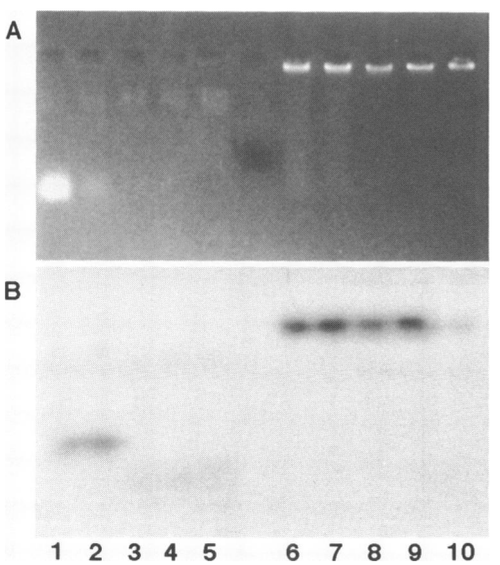

Figure 3. Representative electrophoretic gels of DNA, incubated in fresh rat serum. Samples were visualized by ethidium bromide, panel **A**; and by autoradiography, panel **B**. Lanes 1–5 contain oligonucleotide alone; lanes 6–10 contain oligonucleotide in complex. Lanes 1 and 6 were incubated for 0 hours; 2 and 7 for 1 hour; 3 and 8 for 2.5 hours; 4 and 9 for 5 hours; and 5 and 10 for 24 hours.

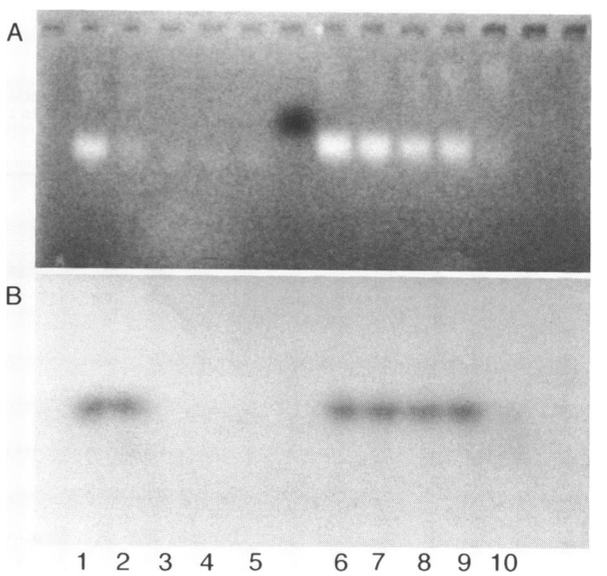

Figure 4. Representative electrophoretic gels of DNA, incubated in fresh rat serum then treated with heparin prior to electrophoresis. Samples were visualized by ethidium bromide, panel **A**; and by autoradiography, panel **B**. Lanes 1–5 contain oligonucleotide alone; lanes 6–10 contain oligonucleotide dissociated from complex. Lanes 1 and 6 were incubated for 0 hours; 2 and 7 for 1 hour; 3 and 8 for 2.5 hours; 4 and 9 for 5 hours; and 5 and 10 for 24 hours.

autoradiogram paralleled the results visualized with ethidium bromide staining.

In contrast to the results observed with serum, no significant differences were seen in the degradation of free or complexed oligonucleotides incubated in crude cell lysate (Fig. 2C and D). The modest amounts of degradation observed with free oligomer suggests that our cell lysate preparations contained less nuclease activity than serum. It is possible that under these conditions inhibition of degradation may not have been readily apparent. It is also possible that, notwithstanding the low level of nuclease activity, components within the lysate, such as lysosomal enzymes, rendered the conjugate ineffective in protecting complexed DNA.

Inhibition of plasmid DNA degradation by conjugate

Cesium purified, double-stranded plasmid DNA was bound to AsOR-PL conjugate and incubated under similar conditions to single-stranded oligonucleotide. In order to maintain the supercoiled physical state of the DNA, we elected not to directly introduce a label into the purified plasmid. Instead, Southern blot hybridization after dissociation of plasmid from conjugate following incubation was employed. Figure 5 shows the

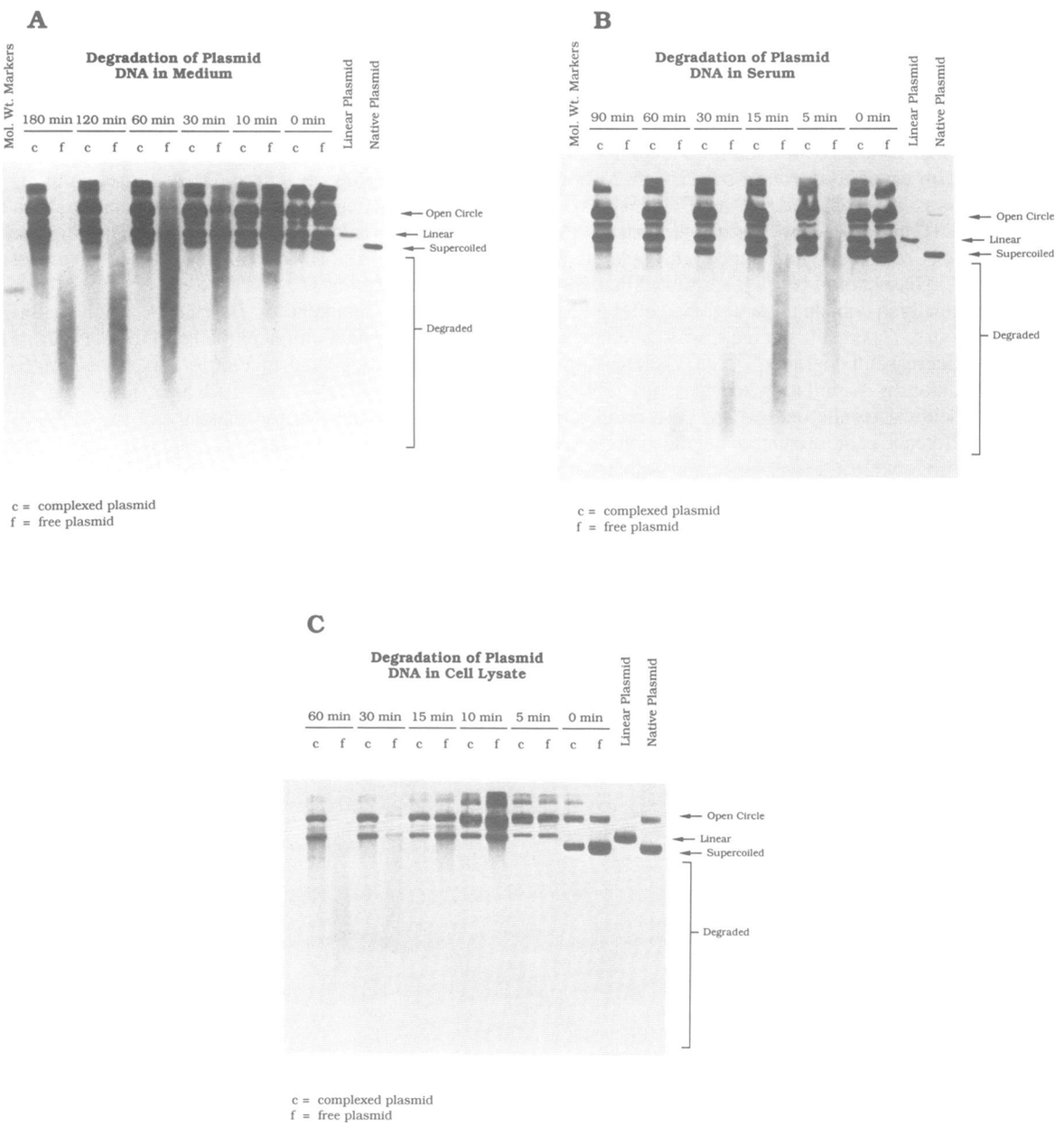

Figure 5. Representative autoradiograms from Southern blots of plasmid DNA incubated in **A**: culture medium containing 10% FBS; **B**: 100% rat serum; **C**: cell lysate. In each panel f = free plasmid, and c = complexed plasmid. The time of incubation is listed above the lanes, and control samples showing the positions of supercoiled, open circular, and linear plasmid is marked on the right of each blot. The region below the position of supercoiled plasmid used to quantitate degraded plasmid is also marked.

autoradiograms of blots of free and complexed plasmid incubated in culture medium, fresh rat serum, or crude cell lysate. The positions of supercoiled, open circular, and full-length linear forms are marked. All hybridizing species that migrated faster than supercoiled plasmid were considered to be degraded DNA. The blots show that, under all conditions, free plasmid was degraded more rapidly than plasmid bound to AsOR-PL conjugate. The results of densitometric quantitation of the autoradiograms are presented in Table 1 as percentage remaining after incubation compared to the zero time point. After 3 hours of incubation in tissue culture medium the amount of complexed supercoiled plasmid decreased about 20% with a concomitant increase in degraded DNA (Table 1A). Open circular and linear forms of the plasmid showed no decrease at all, either individually or grouped together. By comparison less than 10% of free plasmid remained in any of the three full-length forms after 2 hours of incubation.

As expected, degradation was even more rapid in fresh serum. Greater than 90% of free plasmid was degraded to less than full-length fragments within 15 minutes (Table 1A). When complexed to AsOR-PL conjugate, the amount of open circular and full-length linear DNA remained nearly constant over 90 minutes of incubation. The amount of the supercoiled form did decline over time, from 80% of the initial amount remaining after 15 minutes to 23% of initial after 90 minutes. These results indeed show that binding of AsOR-PL conjugate to double-stranded DNA significantly inhibited their degradation by serum nucleases.

To approximate intracellular degradation of plasmids, crude cell lysates were used. Interestingly, the supercoiled plasmids disappeared rapidly, whether or not DNA was bound to conjugate (Figure 5C, Table 1B). The results suggest that there was a high level of DNA nicking activity present in our cell lysate preparations, since a single nick on either strand would convert a supercoiled plasmid to open circular form. At early times during the incubation there was an accumulation of open circular molecules with both complexed and free plasmid. At later time points however, the open circular and linear forms of the free plasmid disappeared while these forms either remained constant or continued to accumulate in incubations with complexed plasmid. The results suggest that although initially it appeared that complexed and free plasmids were being nicked at similar rates, longer time points showed that plasmid bound to conjugate, though converted to open circular and linear forms, was still being degraded at a slower rate than free DNA.

DISCUSSION

The stability of oligodeoxynucleotides has been studied previously in a variety of medium, such as reticulocyte lysates (6, 13), HeLa cell lysates (14), culture medium (6) and serum (5–7).

Table 1. A: Change in the amounts of supercoiled (SC), open circular (OC) plus linear, and degraded plasmid DNA after incubation in culture medium or serum for various times

Condition		Complexed Plasmid DNA			Free Plasmid DNA		
	Incubation time (min)	SC	OC & Linear	Degraded	SC	OC & Linear	Degraded
MEM	0	1.0	1.0	1.0	1.0	1.0	1.0
w/ 10% FBS	10	.9	1.1	1.4	.9	1.3	16.1
	30	.9	1.2	1.6	.8	1.1	23.2
	60	1.0	1.2	2.7	.7	.8	50.7
	120	.9	1.2	3.2	.1	0	32.0
	180	.8	1.3	4.8	0	0	22.8
Serum	0	1.0	1.0	1.0	1.0	1.0	1.0
	5	.8	1.3	.9	.2	.3	2.9
	15	.8	1.3	1.4	.1	.1	6.1
	30	.5	.9	1.2	0	0	2.8
	60	.4	1.0	1.0	0	0	1.3
	90	.2	1.0	.8	0	0	.6

For each DNA species the ODxmm^2 densitometric quantitation at each time point was divided by the amount of that species present at zero time to show the change that occurred (i.e. the numbers are arbitrary units based upon the amount of DNA present at time zero). To simplify the table the results of the open circular and linear DNA calculations were combined since these two groups were similar at each time point. These results are from one representative experiment. Similar results were observed in three additional experiments using serum and two additional experiments using culture medium.

Table 1. B: Change in the amounts of supercoiled (SC), open circular (OC), linear, and degraded plasmid DNA remaining after incubation in cell lysate from one representative experiment

Condition	Incub-ation time (min)	Complexed Plasmid DNA				Free Plasmid DNA			
		SC	OC	Linear	Degraded	SC	OC	Linear	Degraded
Cell Lysate	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	5	0	1.9	14.6	2.0	0	1.4	3.6	1.0
	10	0	1.8	26.2	4.0	0	2.7	9.9	33.0
	15	0	1.1	22.0	29.0	0	1.7	6.7	146.0
	30	0	1.3	30.3	347.0	0	.2	2.2	2095.0
	60	0	1.3	32.9	1801.0	0	.1	.8	3893.0

Similar results were observed in two additional experiments. Results are presented as described above.

Depending upon incubation conditions and method used to assess degradation, half-lives of DNA have been reported to be anywhere from several minutes to several hours. Our results confirm that single-stranded oligodeoxynucleotides and double-stranded DNAs are susceptible to degradation in these media. In addition, our data demonstrates that AsOR-PL-DNA complexes do not readily dissociate under any of the conditions tested, and that formation of these complexes enhances resistance to degradation for both single- and double-stranded DNA. This may, at least in part, be due to the demonstrated ability of polylysine containing conjugates to condense DNA (15). When condensed, as in the case of chromatin, the DNA may be less exposed to nucleases. Protection of oligonucleotides may principally be a function of shielding the 3'-terminus from exonucleolytic activity. In published experiments, Boado and Pardridge (16) showed that coupling of avidin to near-3'-biotinylated 21-mer oligonucleotides resulted in protection whereas avidin binding conferred no nuclease resistance when the biotin was incorporated into the 5'-end of the oligonucleotide. One advantage of polylysine conjugates is that it does not have to be covalently coupled to DNA as in the case of biotin–avidin mediated protection.

In comparison to single-stranded oligonucleotides, free double-stranded supercoiled plasmid DNA was much more rapidly degraded under all conditions, particularly in serum. The extreme sensitivity of naked plasmid DNA to damage and degradation highlights how advantageous it can be to have a carrier system that protects DNA from nuclease attack. Previous studies have shown that within 10–20 minutes of intravenous administration 80–85% of the total amount of AsOR-PL-DNA complexes injected is found within the liver, 80% of which was localized specifically to hepatocytes (3, 17). Our studies with plasmid DNA incubated in serum show that in the above time frame for intravenous delivery, the vast majority of plasmid bound to AsOR-PL conjugate remained intact. Naked plasmid, on the other hand, was greater than 90% degraded over that same time period. Without protection from nuclease degradation very little of the DNA would arrive at the target cells *in vivo* in a functional state.

It is interesting to note the differences in the degradation pattern of plasmid DNA in serum versus crude cell lysate. Incubations in cell lysates resulted in a rapid accumulation of nicked-circular forms. Linear molecules, probably converted from circular forms by multiple single-stranded nicks occurring on opposite strands in proximity to each other, transiently increased in amount before being further degraded in cell lysate. In serum, linear forms were much more rapidly degraded, without any accumulation. This suggests that there is a higher level of exonucleolytic activity in serum compared to cell lysate. Our results with oligomers suggest that double-stranded DNA was more susceptible than single-stranded forms.

Examination of the degradation pattern in cell lysates suggests that the protein conjugate is effective in protecting DNA from exonucleases, but less able to protect from endonucleolytic nicking. However, the high level of nicking activity may have been due, at least in part, to the addition of DNase I to the preparations (see Materials and Methods). Most of the damage sustained by plasmid bound to AsOR-PL was in the form of nicks that converted the DNA to open circles and linear molecules. Even after 60 minutes of incubation in cell lysate a large proportion of complexed plasmid remained full-length in one of those two forms. Thus, the results we observed may substantially overestimate the amount of degradation caused by endogenous nucleolytic activity. Nevertheless it is also possible that proteases and degradative enzymes released from lysosomes and other intracellular organelles diminished the conjugate's ability to protect DNA. This possibility further supports a strategy of avoiding exposure to lysosomes by actively mediating the release of DNA into the cytoplasm immediately after endocytosis. Indeed, the use of endosomolytic substances for this purpose has been already shown to substantially improve levels of targeted gene expression (18–20).

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