Selective enhancement of gene transfer by steroid-mediated gene delivery

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The incorporation of transgenes into the host cells' nuclei is problematic using conventional nonviral gene delivery technologies. Here we describe a strategy called steroid-mediated gene delivery (SMGD), which uses steroid receptors as shuttles to facilitate the uptake of transfected DNA into the nucleus. We use glucocorticoid receptors (GRs) as a model system with which to test the principle of SMGD. To this end, we synthesized and tested several bifunctional steroid derivatives, finally focusing on a compound named DR9NP, consisting of a dexamethasone backbone linked to a psoralen moiety using a nine-atom chemical spacer. DR9NP binds to the GR in either its free or DNA-crosslinked form, inducing the translocation of the GR to the nucleus. The expression of transfected DR9NP-decorated reporter plasmids is enhanced in dividing cells: expression of steroid-decorated reporter plasmids depends on the presence of the GR, is independent of the transactivation potential of the GR, and correlates with enhanced nuclear accumulation of the transgene in GR-positive cells. The SMGD effect is also observed in cells naturally expressing GRs and is significantly increased in nondividing cell cultures. We propose that SMGD could be used as a platform for selective targeting of transgenes in nonviral somatic gene transfer.

Using nonviral methods of gene transfer, only a small portion of the delivered nucleic acids undergoes nuclear translocation, especially in nondividing cells[1–6]. One solution is to employ viral vectors: viruses have evolved mechanisms to deliver their genes into the nuclei of host cells, explaining the higher transduction efficiency achieved using viral vectors[7,8]. A surrogate strategy for overcoming the barrier of the nuclear envelope is to link oligopeptides encoding a nuclear localization signal (NLS) to the transgene DNA[9–11]. Here we propose an alternative strategy, SMGD, which enhances the translocation of the transgene into the nucleus (Fig. 1A). The rationale behind SMGD is to facilitate the nuclear uptake of transfected DNA with the help of nuclear receptors such as the GR, which actively migrates to the nucleus once activated by its steroid ligand[12–15].

The essential reagents for SMGD are derivatives of steroids that can be tethered to transgenic DNA. The steroid-decorated transgenes are introduced in the cell by conventional transfection procedures (Fig. 1A, step 1) and are subsequently transported to the nucleus by receptor interaction (steps 2 and 3). For this study, we modeled the SMGD approach using the well-characterized GR system [12,16,17]. Our work demonstrates that both the expression and the nuclear uptake of DR9NP-decorated reporter plasmids are selectively enhanced in the presence of GR. We suggest that nuclear receptors can be exploited as intracellular gene transfer vehicles.

Results

Structure and properties of DR9NP. We synthesized and tested a variety of hormone spacer–DNA binder conjugates in which we intended to maintain the hormones' affinity for the GR and its capacity to trigger GR's nuclear translocalization (A.B. and A.R., unpublished data; ref. 18). Our investigations revealed that the

dexamethasone–psoralen conjugate DR9NP (Fig. 1B) was the most suitable compound. In DR9NP, the receptor-binding (dexamethasone) and the DNA-binding (psoralen) function are separated by a nine-atom chemical spacer flanked by urethane bonds, providing resistance to degradation (see Experimental Protocol).

In Figure 1C we compare the affinity of DR9NP (open circles) and dexamethasone (filled circles) in a receptor-binding competition assay. In this assay we find that the equilibrium binding affinity constant of DR9NP (K_2, 7.95×10^{-8} M) is about 16-fold higher than that of dexamethasone (K_1, 6×10^{-9} M). In Figure 1D we show that DR9NP-crosslinked plasmid DNA is preferentially immunoprecipitated when incubated with GR (filled bars) compared with uncrosslinked plasmid (hatched bars). The preferential immunoprecipitation of DR9NP-crosslinked DNA, but not of naked DNA, is inhibited by dexamethasone, indicating that the preferential interaction is mediated by the ligand-binding pocket of GR. Figure 1E–G show that free DR9NP is able to induce nuclear translocation of a GR–green fluorescent protein (GFP) chimera. Fluorescence micrography revealed that a transiently expressed GR(Ala)GFP chimera is normally localized in the cytoplasm (Fig. 1E), whereas in the presence of 500 nM dexamethasone (Fig. 1F) or 500 nM DR9NP (Fig. 1G), the chimeric protein accumulates in the nucleus.

Therefore, DR9NP fulfills the criteria of stability, affinity, and ability to induce nuclear translocation, all of which are necessary to test its ability to enhance the nuclear import and expression of a tethered reporter transgene. To enhance the transfection experiments, we used the constructs illustrated in Figure 2: LacZ and control reporter genes are indicated in Figure 2A, and the recombinant adenoviral vectors encoding various GR and control constructs are shown in Figure 2B.

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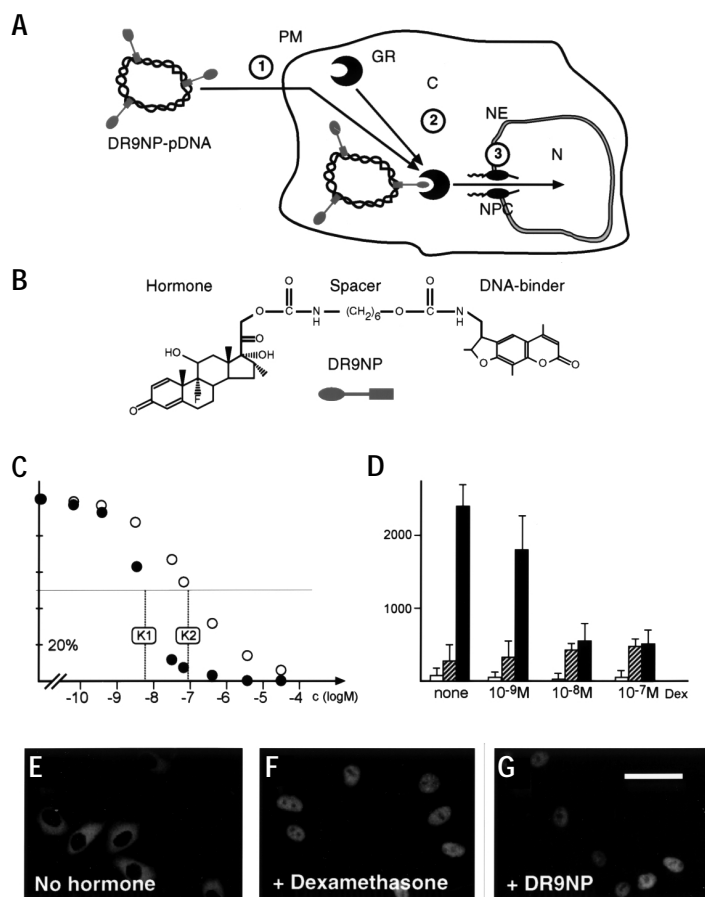
Figure 1. Structure and properties of the bifunctional steroid derivative, DR9NP. (A) Cellular events involved in SMGD. DR9NP molecules (see panel B) are crosslinked to plasmid DNA (pDNA). The resulting complex DR9NP–pDNA is transported into the cytoplasm by conventional transfection (step 1). Glucocorticoid receptor (GR) located in the cytoplasm binds to the steroid moiety of DR9NP–pDNA (step 2). Steroid recognition results in nuclear translocation of GR and associated DR9NP–pDNA (step 3). C, Cytoplasm; PM, plasma membrane; NE, nuclear envelope; NPC, nuclear pore complex; N, nucleus. (B) Structure of bifunctional (steroid)–spacer–(DNA binder) compounds. DR9NP is composed of dexamethasone linked by urethane bonds to a nine-atom spacer to the DNA-crosslinking molecule psoralen. (C) Receptor-binding assay of dexamethasone and DR9NP. Cytosolic fractions containing GR were incubated with tritiated dexamethasone and competed with the amounts of cold ligand indicated in the x-axis. The filter-bound counts were expressed as a percentage of the uncompeted samples (y-axis). (●, dexamethasone competitor; ○, DR9NP competitor. The relative K_D values were calculated on a Scatchard plot. In the figure the positions of the K_D for dexamethasone ($K_1 = 6 \times 10^{-9}$ M) and for DR9NP ($K_2 = 9.5 \times 10^{-8}$ M) are indicated by the vertical dotted lines. Standard deviations of each point are reported in the Experimental Protocol.
(D) Immunoprecipitation of GR–plasmid complexes (see Experimental Protocol). Competitor dexamethasone (Dex) was present in the indicated concentrations. The immunoprecipitated plasmid was measured in a dot-blot hybridization. The y-axis indicated the counts per minute of dots from triplicate experiments. Filled bars, DR9NP-decorated plasmid (preparation D5, described in Fig. 3); hatched bars, naked plasmid; empty bars, reaction without cytosol (background binding to preadsorbed anti-GR antibodies). The four sets of experiments were conducted in the absence (none) or in the presence of competitor dexamethasone (5×10^{-9}, 5×10^{-8}, 5×10^{-7} M).
(E–G) Cellular localization of transiently transfected GR(Ala)GFP in the absence of hormone (E), in the presence of either 5×10^{-7} M dexamethasone (F) or 5×10^{-7} M DR9NP (G). Scale bar: 50 μm.

DR9NP crosslinking to reporter DNA. The reporter plasmid *CMVLacZ* (Fig. 2A, construct 2) was UV-irradiated in the absence or presence of four different concentrations of DR9NP and purified as described in the Experimental Protocol. Portions of the resulting DR9NP-crosslinked *CMVLacZ* preparations (called D8, D7, D6, and D5 and the negative control, D0) were restriction-linearized and analyzed by denaturing gel electrophoresis (Fig. 3A). Under these conditions, D0 migrated exclusively as single-stranded DNA (lane 1), while D8, D7, D6, and D5 produced increasing amounts of double-stranded DNA signals (lanes 2–5). The percentage of crosslinked DNA (open circles in Fig. 3B) and the number of crosslinks (numbers on top of Fig. 3B) were calculated for each reporter preparation. As anticipated, the increase of random crosslinking progressively deactivates the reporter vector as docu-

mented in Figure 3B (filled circles), where we measured the amount of reporter *LacZ* activity in CV-1 cells transfected with equivalent amounts of either preparation. The activity of the preparations D6 and D5 is reduced to 90% and 36%, respectively, and we rationalize that the loss of gene activity is resulting from crosslinking within biologically important regions of the vector (Fig. 3C).

Enhanced expression of steroid-decorated reporter genes. For the fundamental experiments, we chose CV-1 cells, because they do not express active GR (refs.19–21). To supplement CV-1 cells with GR, we used recombinant adenoviruses encoding either a neutral gene (such as GFP), wild-type GR, or the mutant GR(Ala).

Figure 4A shows the time course of the infection/transfection procedure. In the control experiment shown in Figure 4B we demonstrate the effect of adeno-encoded GR on an *MMTV-LacZ* reporter in the presence of dexamethasone (bars 3 and 4). The compilation of independent experiments involving the preparations D0 and D6 is shown in Figure 4C. In the absence of GR, there is no significant difference in the expression levels of the D0 and D6 preparations (Fig. 4C, bars 1 and 3), while in the presence of GR, D6 has a two- to threefold higher expression than either D0 or D6 on the absence of GR (compare bar 4 with bars 3 and 2). In Figure 4D we show that the enhancement of expression can be dissociated from the transactivation capacity of GR by expressing a GR(Ala) mutant. The expression of the hormone-decorated preparations D6 and D5 was enhanced in the presence of either wild-type GR (Fig. 4D, bars 5 and 8) or nontransactivating GR(Ala) (Fig. 4D, bars 6 and 9).

Increased nuclear accumulation of DR9NP-decorated DNA. To directly monitor the cellular localization of the transfected plasmids, we lipofected rhodamine-labeled D0 or D5 preparations in CV-1 cells expressing either GR or negative control products. The trans-

Table 1. Cellular localization of naked and DR9NP-decorated plasmid DNA in presence or absence of wild-type or mutant glucocorticoid receptor

r-Adenovirus[a] Vector	A Mock D0	B GRwt D0	C GR(Ala) D0	D Mock D5	E GRwt D5	F GR(Ala) D5
Cytoplasmic[b]	55 ± 5	53 ± 4	56 ± 7	55 ± 9	39 ± 13	48 ± 6
Perinuclear[b]	40 ± 6	43 ± 4	42 ± 6	41 ± 8	48 ± 14	42 ± 6
Nuclear[b]	4 ± 1	4 ± 1	3 ± 1	4 ± 1	13 ± 4	11 ± 1

[a]The experimental conditions are described in the legend of Figure 5 and in Experimental Protocol.
[b]Rhodamine signals as exemplified in Figure 5 were counted in about 150 cells for each experiment and subdivided in the three classes cytoplasmic/perinuclear/nuclear. The numbers indicate the percentage of the total scored signals for each condition; the total numbers of scored signals were 1,170 for A, 1,151 for B, 976 for C, 901 for D, 809 for E, and 786 for F.
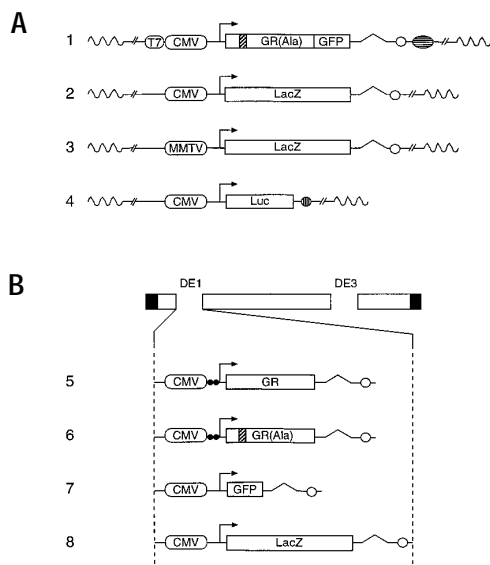
Figure 2. Plasmids and recombinant adenoviruses. (A) Reporter plasmids. The linear maps outline the essential biological elements but are drawn out of scale. Vector names: **1**, CMVGR(Ala)GFP; **2**, CMVLacZ; **3**, MMTVLacZ; **4**, CMVLuc. (B) Recombinant adenoviruses. A scheme of the genome of recombinant Ad5 vectors (not to scale). Vector names: **5**, AdGR; **6**, AdGR(Ala); **7**, AdGFP; **8**, AdLacZ. Symbols and abbreviations: wavy line, plasmid sequence; T7, T7 bacteriophage RNA polymerase promoter; CMV, cytomegalovirus promoter/enhancer; bent arrow, transcription start; hatched box, polyalanine stretch; GR(Ala)GFP, fusion of GR(Ala) and GFP; broken line, rabbit β-globin splice signal; empty circle, rabbit β-globin 3′-polyadenylation signal; hatched oval, SV-40 origin of replication; LacZ, β-galactosidase gene; MMTV, mouse mammary tumor virus inducible promoter; Luc, luciferase gene; hatched circle, SV-40 polyadenylation signal; filled box, adenoviral inverted terminal repeat (ITR); DE1, deletion of adenoviral E1 region; DE3, deletion of adenoviral E3 region; filled circles, 2× tet operators; GR, glucocorticoid receptor gene; GR(Ala), GR dominant negative mutant[33]; GFP, green fluorescent protein gene.

fected samples were stringently rinsed, counterstained with acridine orange, and imaged with a confocal laser-scanning microscope. A representative picture is shown in Figure 5A, where rhodamine-labeled aggregates appear like punctuated red/orange signals. The cellular localization of the signals is quantified in Table 1. In mock-infected cells there is no significant difference in the cellular localization of D0 and D5 (Table 1, columns A and D), and the localization of undecorated DNA does not change on expression of either GRwt or GR(Ala) (Table 1, columns B and C versus A). Instead, the D5 preparation produces a 3-fold higher percentage of nuclear signals in the presence of GRwt (column E versus D, $P < 0.0001$) and 2.5-fold in the presence of GR(Ala) (column F versus D, $P < 0.0001$). The exact localization of dots was verified by analyzing the localization of the nuclear signals by lateral-view reconstruction of the confocal images. (See Figures 5B–D, which show the analysis of the cell boxed in Figure 5A). From either perspective, two orange-yellow signals are clearly localized in the nucleus, and one red signal in the perinuclear region.

SMGD with native GR receptor. To verify whether SMDG can be used to enhance transfection in cells naturally expressing GR, we transfected the cell line A549, widely used to study glucocorticoid effects[22,23]. As shown in Figure 6A, we compared the expression of different amounts of transfected DR9NP-decorated *CMV-LacZ* (D6, filled triangles) and undecorated DNA (D0, filled circles). In this assay the steroid-decorated DNA has a significant advantage that progressively diminishes at higher levels of transfected plasmid
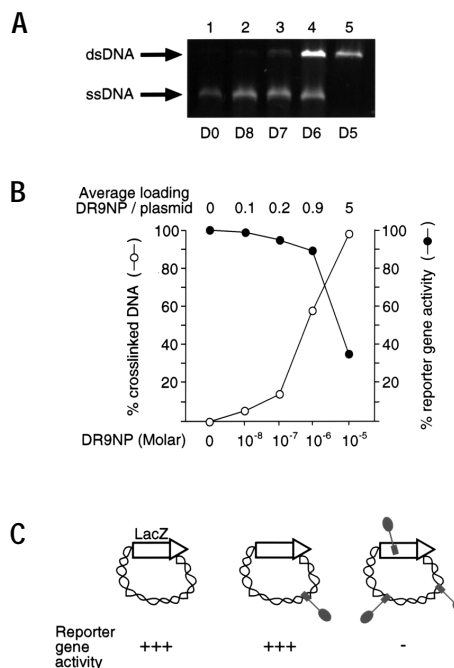

Figure 3. Characterization of DR9NP-crosslinked CMVLacZ. (A) Electropherogram of linearized, alkali-denatured CMVLacZ (10 kb, construct 2, Fig. 2A) after UV-crosslinking in the presence of various concentrations of DR9NP. Under those conditions, crosslinking DNA fragments maintain their double-stranded structure and are visible as slow migrating bands (dsDNA), while uncrosslinked DNA fragments remain single-stranded and are seen as fast migrating bands (ssDNA). The resulting CMVlacZ preparations were named D0 (lane 1, UV irradiation in absence of DR9NP) or D8 through D5 (crosslinked in presence of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M DR9NP, respectively). (B) Analyses of DR9NP crosslinked reporters. Top line: average number of crosslinks per plasmid calculated as described in Experimental Protocol. Graph: fraction of DR9NP-crosslinked CMVLacZ (○, left scale axis) and percentage of β-galactosidase (β-Gal) reporter activity (●, right scale axis). Fraction of crosslink: the intensity of the bands shown in (A) was quantified by densitometry. The percentage of crosslinked DNA was calculated for each vector by dividing the intensity of the dsDNA by the sum of both signals. The average numbers of crosslinks were calculated basing on the Poisson's distribution, where the probability P to have n crosslinks can be defined as $P(n,L) = (L^n/n!) \times e^{-L}$, where L = average number of crosslinks. For $P = 0$, L can be extrapolated as $L = -\ln\{P(0)\}$, where $P(0)$ is the measurable fraction having no crosslinks (ssDNA). The calculation based on crosslinking is probably an underestimation, as psoralen can generate mono-adducts that would not be detectable with this assay[43]. Surviving reporter activity was determined by transiently transfecting CV-1 cells with equal amounts of the different preparations and by measuring the expressed LacZ. The percentages of reporter gene activity have been normalized by comparing each preparations' activity by the one of D0, which is defined as 100%. (C) Illustration of the three classes of possible molecules in crosslinked preparations and rationalization of the progressive loss of biological function with increasing random crosslinking. The three schemes represent the reporter vector without crosslinks (left), with crosslinks outside the reporter gene (center), and with at least one crosslink in the LacZ gene (right). Symbols: +++, active; –, inactive.

because of saturation effects. In parallel, we observed that the SMGD effect could be competed for by free GR agonist (open versus filled triangles) implying that the effect is mediated by GR's ligand-binding domain.

To verify that SMGD is effective in nonproliferating cells, we transfected postconfluent A549 with different amounts of DR9NP-decorated or undecorated *CMVLacZ* reporter (Fig. 6B). As expected, the general transfectability of the nondividing cells was much reduced (note y-axis values). Nevertheless, at subsaturating concentrations of transfected reporter plasmid, the advan-
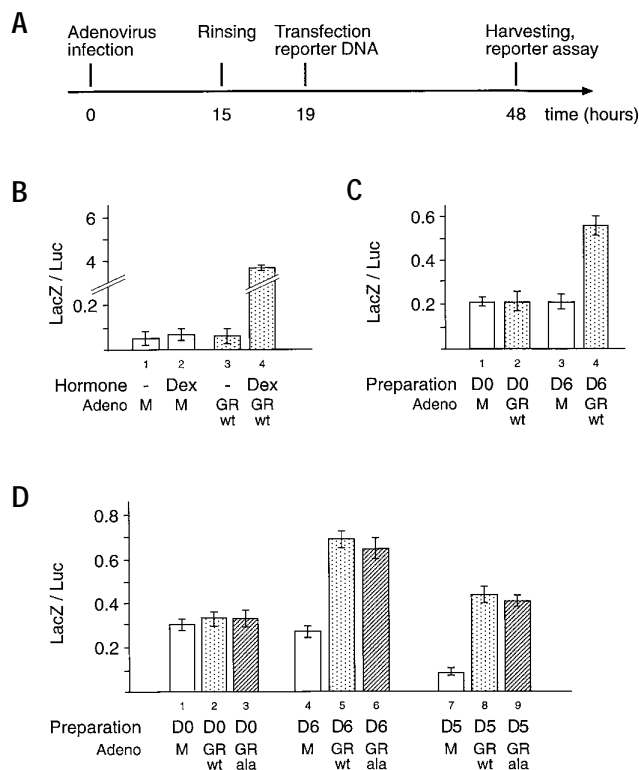
Figure 4. Activity of DR9NP-decorated reporter plasmid is enhanced in presence of GR. (A) Time diagram for the infection/transfection assay. At time point 0 h, CV-1 cells were infected either with AdGFP (Mock) or AdGR, rinsed (15 h), and lipofected (19 h) with 10 ng of either uncrosslinked CMVLacZ (D0) or 10 ng DR9NP-crosslinked CMVLacZ (D6 or D5) supplemented with 100 ng uncrosslinked CMVLuc as internal reference. Cells were harvested (48 h) and the activities of β-Gal and Luc were determined simultaneously. The presence of GR in all the infected cells was assayed by immunodetection and was reached by infection at MOI 14 (data not shown) in a reporter CV-1 derivative that bears a chromosomally anchored MMTV reporter LacZ gene (S. Brenz Verca *et al.*, pers. commun.). (B) Verification of biological activity of the adeno-transduced GR. After infection with either AdGFP (Mock) or AdGR, CV-1 cells were lipofected with 300 ng of GR-inducible MMTVLacZ reporter (Fig. 2A, construct 3) and quantified. Symbols: Dex, dexamethasone (5×10^{-5} M); M, pre-infection with mock r-adenovirus (empty bars); GRwt, pre-infection with r-Adeno expressing wild-type GR (stippled bars). The bar heights represent relative β-Gal activity with standard deviations. (C) Enhancement of reporter activity for the DR9NP-decorated CMVLacZ in the presence of wild-type GR. The pre-infected cells were transfected with 10 ng of either reporter preparation D0 (bars 1, 2) or D6 (bars 3, 4). (D) Comparison of D0, D5, and D6 reporter activities in the absence of GR or in the presence of wild-type GR or mutant GR(Ala) (hatched bars). The transfection cocktail contained 100 ng control reporter (CMV-Luc) and 10 ng of each CMVLacZ plasmid preparation D0, D6, or D5. In (B) and (C) the average (±s.d.) of at least three independent experiments is given, while the data in D are from an individual experiment with triplicate samples.

Figure 5. Bi- and tri-dimensional viewing of nuclear localized plasmid DNA after transfection. CV-1 cells cultured on slides were infected with AdGR and subsequently lipofected with rhodamine-labeled D5 preparation. Fixed cells were stained with acridine orange to visualize nuclei and general cellular structures by confocal laser scanning microscopy. The images are composites of 35 optical sections obtained at 200 nm intervals through the z-axis. Rhodamine-labeled vectors appear like punctuate red signals. (A) Wide-field confocal microscope image. The white rectangle highlights a cell with three signals that overlap with the nuclear area. Other symbols: open white arrow, example of cytoplasmic signal; filled white arrow, example of perinuclear signal; filled yellow arrow, example of nuclear-located signal; white bar, 10 μm scale. (B–D) Enlargement of the cell highlighted in (A). Dashed vertical lines define a 3.5-μm-wide portion that has been rotated clockwise by 90 degrees around the y-axis and projected in (C). Dashed horizontal lines define a 4-μm-wide portion that has been rotated clockwise by 90 degrees through the x-axis and is projected in (D). The thin yellow arrows connect the different projections of one of the nuclear localized signals. The white bar in B represents 5 μm and is valid for (C) and (D).

derivative DR9NP, we demonstrated specific receptor binding, induction of nuclear translocation of receptor, crosslinking to plasmid DNA, and finally enhancement of expression and of nuclear accumulation of steroid-decorated reporter plasmids. The two latter effects depend on the presence of GR but are dissociable from its transactivation potential. The SMGD principle works also with physiological levels of GR, providing a marked advantage for the uptake of hormone-decorated transgenes in quiescent cells. This result is important, proving that even in this prototypic form, the SMGD approach promises to significantly improve transfection under conditions relevant for somatic gene transfer.

SMGD provides significant advantages over other strategies aiming at increasing nuclear uptake. The delivery of NLS-coupled transgenes does not target DNA specifically to a desired cell type, as the factors composing the nuclear import machinery are ubiquitous[24]. The steroid-decorated transgenes proposed here therefore offer a selective advantage, targeting genes to cells expressing a specific nuclear receptor. Other potential targets could be nuclear receptors active in particular cancers[25–28], or the nuclear receptors for retinoic acids, vitamin D, thyroid hormones, and peroxisome proliferators.

In our nuclear accumulation studies we observed a dotted pattern of transgene expression rather than a diffuse one. The same dotted

tage of the D6 preparation over the undecorated D0 is pronounced (between 16- and 40-fold).

Taken together, the results with the A549 cells indicate that the SMGD effect is reproducible and can be competed for using ligand in cells expressing physiological concentrations of GR, and that the SMGD effect is magnified in nondividing cells.

Discussion

We have shown here that SMGD can exploit nuclear receptors as ferries for plasmid DNA. We used glucocorticoid receptors as a model system because of the ready availability of expression vectors, receptor mutants, and suitable ligands for derivatization. Using the steroid
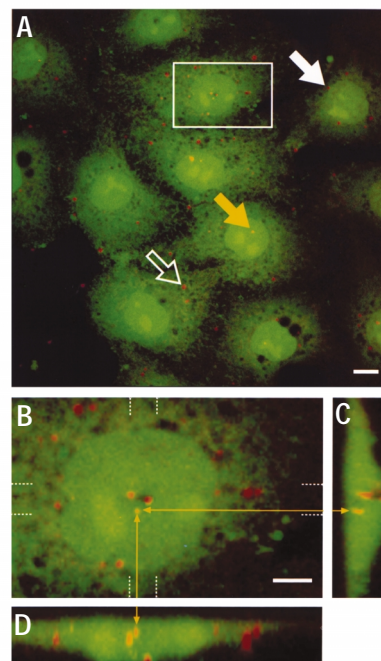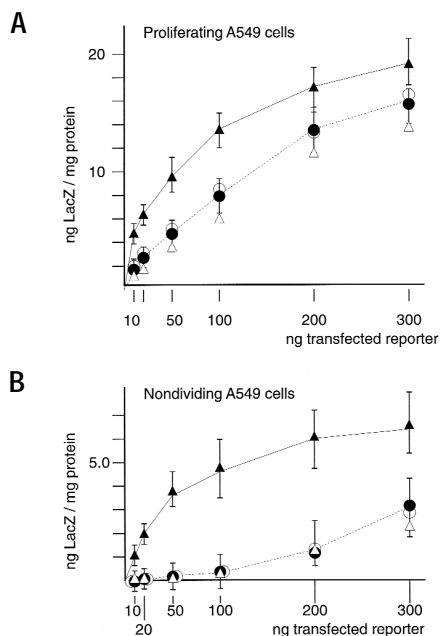
Figure 6. Enhancement of transfection through SMGD in (A) proliferating and in (B) nondividing A549 cells. The A549 cell culture conditions and composition of the liposomal complexes are specified in Experimental Protocol. The proliferating status of the A549 cells was verified by tritiated thymidine incorporation of parallel cultures. In these assays, postconfluent cells (B) gave less than 1% thymidine incorporation than proliferating ones (A). The SMGD effect (A) is observed in A549 cells and is inhibited by addition of competitor ligand. The graph represents the levels of normalized LacZ (y-axis) from different extracts, as a function of the transfected reporter plasmid DNA in the cocktail (x-axis). The lacZ levels were evaluated by comparison to a standard curve obtained with commercial β-galactosidase and normalized with the internal control (co-transfected luciferase reporter). A549 cells were transfected in triplicate with increasing amounts of D6 (▲) or undecorated reporter (●). In parallel, triplicates of each transfection were conducted in the presence of 50 nM dexamethasone (open symbols). Connecting line and standard deviations are given only for the experiments without competitor hormone. The ratios of the expression levels for decorated (D6) versus undecorated DNA (D0) were (in the order from 10 to 300 ng transfected plasmid) 2.9-, 2.5-, 2.2-, 1.7-, 1.3-, and 1.2-fold, respectively. (B) The SMGD is more pronounced in nondividing cells. The representation and the symbols are as in (A). Postconfluent A549 cells were transfected as in (A). Some symbols were laterally displaced to better show their y-value. The ratios of the expression levels D6 versus D0 were (in the order 10 to 200 ng) 43-, 26-, 16-, 13-, and 6.6-fold, respectively.

appearance is seen in the preparation before transfection (unpublished observations). Furthermore, we believe that the 0.2–0.8 μm fluorescent dots are fully compatible with single plasmid signals; there are enough fluorophores per plasmid in our preparation to ensure detection, and the relaxed form of the 10 kilobase plasmid *CMVLacZ* would have a diameter of 1 μm. Similar punctuated signals have been reported for nuclear-imported plasmids in digitonin-permeabilized cells and for HIV-1 genomes in the nuclei of infected cultured cells[29,30]. By contrast, diffuse staining was observed for DNase I-treated DNA, oligonucleotides, and NLS-bearing proteins[29]. Thus, we believe that the diffuse nuclear staining observed in some reports[31] was probably caused by the nuclear import of degraded nucleic acids or recycled fluorescent markers rather than intact plasmids.

In conclusion, this study provides proof of the principle behind the SMGD approach. Future investigations could improve the reagents used, and assess whether the principle can be extended to other nuclear receptors. Furthermore, SMGD is a very promising strategy for clinical applications aiming at the selective macromolecular treatment of target tissues that express a specific steroid receptor.

Experimental protocol

Construction of reporter plasmids and r-adenoviruses. *Reporter plasmids.* The plasmid CMVGR(Ala)GFP (Fig. 2A, construct 1) was constructed by fusing a mutant GFP(S65T) at the C terminus of a mutant rat GR (refs 32–34). Plasmids CMVLacZ (Fig. 2A, construct 2) and MMTVLacZ (Fig. 2A, construct 3) carry an expression cassette for β-galactosidase (LacZ) driven by the human cytomegalovirus (hCMV) promoter and mouse mammary tumor virus (MMTV) promoter, respectively. Plasmid CMVLuc (Fig. 2A, construct 4; gift of M. Imhof) constitutively expresses firefly luciferase. All the plasmids were purified by CsCl.

Recombinant adenoviruses. All recombinant adenoviruses were generated as cloned, full-length recombinant genomes (referred to as "virusmids") in *Escherichia coli* from a *Pac*I-flanked, E1/E3-deleted adenovirus type 5 backbone (vmRL-CMV1; S. Brenz Verca *et al.*, pers. commun., modified from ref. 35). AdGR (Fig. 2B, construct 5) and AdGR(Ala) (Fig. 2B, construct 6) were generated by recombining vmRL-CMV1 with the corresponding expression vectors for wild-type and Ala-mutant GR, respectively. The AdGFP (Fig. 2B, construct 7) is described in Heider *et al*[36]. AdLacZ (Fig. 2B, construct 8) was assembled analogously onto virusmid vmRL-CMV1. Viruses were propagated and purified by standard methods[37]. The lysates were titrated by an endpoint cytopathic assay, aliquoted, and stored at –70°C (refs 38,39).

Synthesis of bifunctional steroid DNA binders. Details on the syntheses of the steroid derivatives are given in the patent entitled "Conjugates of DNA interacting groups with steroid hormones for use as nucleic acid transfection agents"[18]. To generate DR9NP, we derivatized at position 21 the synthetic glucocorticoid dexamethasone because of its superior affinity for the GR. The urethane bond connecting the chemical spacer conferred stability under acidic and basic conditions and resisted protease activities (A.B. and A.R., unpublished data)[18]. The "steroid-spacer" intermediates were further incubated with 4′-aminomethyl trioxalene hydrochloride and *N*-methylmorpholine in tetrahydro-furane (THF) to obtain the steroid spacer–psoralen conjugate DR9NP. All products were analyzed by [1]H- and ^{13}C-nuclear magnetic resonance (NMR). Intermediate and final compounds were tested for receptor affinity and capacity of inducing nuclear transfer of a GR–GFP chimera (described below).

Binding assays. *Free-ligand-binding assays.* The *in vitro* competition assay for binding of hormones or steroid conjugates to GR was done as described[20]. For the binding assays shown in Figure 1C, a cytosolic extract of 4×10^7 3Y1 cells was incubated in the presence of 4.5×10^{-9} M ^{3}H-dexamethasone (Amersham Buckinghamshire, UK) and increasing amounts of unlabeled steroid conjugate competitor.

Crosslinked-ligand binding assay. The GR-binding assay for DR9NP-decorated DNA was done by incubating the cytosol of A549 cells with either undecorated or DR9NP-decorated preparations. Naked plasmid or DR9P-crosslinked plasmids were incubated with cytosolic GR and the complex immunoprecipitated with an anti-GR antibody[40]. In each assay, the cytosol equivalent of 5×10^6 A549 cells was incubated with 20 ng of preparation CMVLacZ D0 or D5 (see Fig. 3). The binding buffer included 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, 150 mM NaCl, and 1 μg competitor calf thymus DNA, for a final volume of 100 μl. Competitor dexamethasone was present in the concentrations indicated in Figure 1D. The reaction was incubated for 30 min at 15°C, after which it was immunoprecipitated with 100 μl Staph-A-preadsorbed antibodies, prepared by mixing 10 μl of ascites supernatant of the anti-GR antibody BuGR2 (a gift of B. Gametchu)[41] with 500 μl Pansorbin/Staph-A cells (Calbiochem, La Jolla, CA). After rinsing, the preadsorbed BuGR2-Staph-A slurry was resuspended in 5 ml binding buffer. The final pellet was resuspended in 50 μl 0.5 M NaOH, 1 M NaCl. The insoluble debris was removed by centrifuging, and 10 μl of each supernatant were spotted onto Nitrocellulose (Schleicher & Schuell PA85, Dassel, Germany). The filters were fixed and hybridized with ^{32}P-labeled CMVLacZ according to standard protocols[42]. The spots were excised and Cerenkov-counted in a beta counter (Tricarb 2000 CA, Canberra-Packard, Zürich, Switzerland). The immunoprecipitated plasmid was measured in a dot-blot hybridization.

Preparation of DR9NP-crosslinked DNA. DR9NP was resuspended in ethanol at a concentration of 10^{-3} M. The different irradiation cocktails containing 25 μg/ml of DNA and, respectively, 0, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M

DR9NP, were prepared in a final volume of 4 ml TE (10 mM Tris-HCl, 1 mM EDTA) (six-well tissue culture plate). The plate was ice bottom-cooled and covered with the plastic lid inside a crosslinker apparatus (BIO-LINK, BLX; Vilber Lourmat, Torcy, France with four UV lamps T-8.L, l = 365 nm) for 1 h, giving a UV intensity of 12 J/cm^2. The crosslinked products were extracted twice with chloroform:isoamyl alcohol (24:1), precipitated with ethanol, and resuspended in TE. For detection of crosslinking, samples were linearized by restriction enzyme digestion, alkali-denatured, and analyzed by agarose gel electrophoresis (see Fig. 3 legend).

Cell culture, infections, transfections, and reporter assays. *Cell culture.* HeLa cells and CV-1 cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 2.5% FCS, 2.5% newborn calf serum, 1% penicillin/streptomycin, and 1% L-glutamine. Cells were maintained at 37°C in a 5% CO$_2$ 100% humidified air atmosphere.

Adenoviral infections. 100,000 CV-1 cells per well were seeded in 24-well tissue culture plates to reach 60–80% confluence during transfection. At time point 0 h (Fig. 4A), cells were incubated with DMEM (3% FCS) supplemented with adenoviral particles to reach a multiple of infectivity (MOI) of 14. At time point 15 h, cells were rinsed twice with Tris-buffered saline (TBS) and subsequently transfected.

Transfection of CV-1 cells. At time point 19 h, cells were lipofected with the transfection reagent TRANSFAST (Promega, Madison, WI) with a charge ratio of 1:2 (6 μl of the reagent to 1 μg of DNA). To each well were added 200 μl of the transfection cocktail, including 10 ng *CMVLacZ* and 100 ng *CMVLuc* and carrier DNA to reach 1 μg. In the samples that included more than 10 ng LacZ reporter plasmid, the amount of carrier DNA was proportionally diminished. After 1 h, the cells were rinsed twice with TBS and supplemented with prewarmed complete medium.

Transfection of A549 cells. For proliferating cells assays, 300,000 A549 cells/well were seeded in 10% FCS DMEM in 12-well dishes. The cells were rinsed and supplemented 4–6 h later with 1.5 ml serum-free medium. The liposomal complexes were added to the wells and incubated for 4 h in either the absence or the presence of competitor hormone (dexamethasone, 5×10^{-8} M). The wells were rinsed and incubated further in 3% FCS DMEM. In the appropriate samples, competitor hormone was maintained for a further 6 h, then removed by rinsing and resupplementing of prewarmed medium. The transfection of nondividing cells was carried out under the same conditions except that the seeded cells were allowed to reach confluency (24 h) and maintained in this status for an additional 24 h before transfection. The transfection cocktail contained a total of 2 μg DNA, composed of 1 μg carrier DNA, 0.5 μg CMVLuc, and variable amounts of either D6 or D0 CMV-LacZ preparation. The variable amounts of LacZ reporter were compensated with CMV-CAT to give a total of 0.5 μg. The DNA cocktails were added to 500 μl serum-free medium to which 10 μl of DOTMA (a gift of the Institute of Molecular Biology, University of Zürich, Switzerland, UNIZH 1 mg/ml in sterile water) were subsequently added.

Proliferation assay for A549 cells. Cultures equivalent to the transfected samples were prepared onto 5 cm dishes and tested for proliferation by [^{3}H]thymidine incorporation. For the labeling, the dishes were rinsed three times with TBS, then covered with 3 ml serum-free DMEM supplemented with 2 μCi [^{3}H]thymidine (NEN Life Science Products, Boston, MA; no. NET-027Z) for 2 h (37°C, 5% CO$_2$). The dishes were then rinsed three times and further incubated 1 h in serum-free medium (same conditions). The cells were rinsed with progressively colder TBS, then exposed to 1 ml ice-cold 5% trichloric acid (TCA). After 30 min at 4°C the TCA was aspirated and the dishes were further rinsed with ice-cold TBS. The cells were then dissolved in 0.5 ml of 0.5 M NaOH/0.5% sodium dodecyl sulfate, (wt/vol)and 200 μl of the solution were counted by mixing with scintillation cocktail. The average counts of the triplicate assays are given in the legend of Figure 6.

Reporter assays. Cells were harvested 24–26 h after rinsing the transfection cocktail, and the activities of β-galactosidase (LacZ) and luciferase (Luc) were determined simultaneously with the chemiluminescent gene reporter assay Dual Light (Tropix; Perkin Elmer–Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The LacZ values were normalized with the values obtained in the Luc assay.

Nuclear translocation assay. CV-1 cells were seeded at 20–30% confluence into 5 cm tissue culture dishes and transfected overnight with a CaPO$_4$ cocktail comprising 2 μg CMVGR(Ala)GFP, 0.5 μg of an expression vector encoding simian virus 40 (SV-40) T-antigen, and 10 μg of carrier DNA. The cells were rinsed twice with TBS, supplemented with fresh medium, and further incubated under standard conditions. Cells were supplemented 24 h after rinsing with the selected ligands at different concentrations. Cells were fixed 4 h later with 2% formaldehyde/0.2% glutaraldehyde and inspected in the presence of UV light with a Zeiss Axiovert fluorescence microscope.

DNA-labeling, confocal microscopy, and image analysis. Naked and DR9NP-decorated CMVLacZ plasmids were labeled with rhodamine using the Label-It reagent (Panvera; Madison, WI) following the manufacturer's instructions (50 μl of DNA at concentration 0.1 mg/ml incubated with 50 μl of the labeling reagent for 1 h at 37°C and purified by Sephadex G50 column). CV-1 cells were lipofected with the rhodamine-labeled vectors as described above; 12 h after transfection the cells were trypsinized and seeded onto coverslips. After adherence, the cells were fixed with 4% formaldehyde and examined with a Bio-Rad 1024, argon/krypton confocal laser scanning fluorescence microscope (LSCM) with K1 and K2 dual filter blocks (Bio-Rad Laboratories, Hercules, CA). Low-magnification (20×), central optical sections of 500 nm were taken from random fields. The red-dotted signals from the pictures were counted as reported in Table 1. In this table the values are presented as mean ± s.d. To visualize nuclei and general cellular structures, cells were counterstained with acridine orange. Acridine orange signals were detected at 605 nm and rhodamine signals at 680 nm. Collected Z-series optical sections were superimposed using the Laser-Sharp-Processing software (Bio-Rad) to visualize the reconstructed three-dimensional images. No further filters or image corrections were applied to the images.

Statistics. The significance statements (related to Fig. 4 and Table 1) were supported by a Welch *t*-test for independent means. Standard deviations were calculated when at least three independent measures were available.

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