Supporting Information: Validation of SR/SRE FP assay

Optimization of salt, detergent, and reducing agent concentrations to eliminate nonspecific DNA binding. A single concentration of hPR or hGR was used to compare FITC-G2T and FITC-random binding to the SRs at various salt concentrations. As shown in Fig S1, physiological ionic strength (150mM NaCl) is the optimum salt concentration that allows substantial binding of hPR to the sequence-specific FITC-G2T, without measurable binding to the random sequence (FITC-random). Results were similar with hGR (data not shown) when 7.5 mM CHAPS was added to the reactions (see below).

Multipoint binding curves were then done at 150mM NaCl to confirm the single point results, and these data are presented in Figure S2 for the hPR. While the hPR interacts very little with FITC-random even in the absence of detergent or reducing agent, there is a small but detectible interaction between FITC-Random and hGR (data not shown). Because the addition of the detergent CHAPS to the hGR assays eliminates this small amount of nonspecific binding (data not shown), CHAPS was included in all FP experiments with the hGR. Inclusion of CHAPS has little effect on hPR binding curves, demonstrating that detergent is not necessary to preclude non-specific DNA binding in our assay system with hPR. Thus, As experiments (see main text) with hPR were performed both with and without CHAPS and confirmed that the detergent does not alter As's effect on binding.

We also needed to be able to perform the FP assay both with and without reducing agent not only to check the effect of this reagent on the assay but also to evaluate any possible alteration of the As response by reducing agent. The hPR is the more robust of the two receptors in terms of its need for reducing agent whereas hGR requires a reducing agent for optimal purification and storage (and, hence, all FP assays with hGR were performed in the presence of GSH). GSH was chosen in part because it is normally found in cells and is at least 1mM in liver-derived cells such as EDR-3. The effect of 1mM GSH on hPR binding to FITC-G2T is shown in Fig S3. While GSH does reduce the K_d by about 5-10 nM, both curves show saturation and equivalent maximal binding, suggesting that the assay is viable with or without GSH. Thus, As experiments (see main text) with

hPR were performed both with and without GSH and confirmed that the reducing agent does not alter As's effect on binding.

Specificity of steroid receptor binding to GRE DNA sequence. Figure S4 shows that the specificity of hGR (Panel A) and hPR (Panel B) binding to the GRE sequence was established by competition experiments (Figure S4). Although the hGR is more sensitive than hPR, there is marked competition for both receptors by unlabeled G2T at all ratios. This contrasts with the random oligonucleotide, where even a 100-fold molar excess is unable to reduce binding of FITC-G2T to either hGR or hPR, further demonstrating the specificity of the receptors' binding interaction with G2T.

Although the receptor preparations were greater than 90% pure, we confirmed that the observed increase in polarization is due to receptor binding by performing immunoprecipitation experiments. FIGR antibody was used to remove hGR from purified preparations as described in the experimental section. Figure S5 compares the FIGR antibody to nonspecific mouse immunoglobulin and shows a substantial mP signal due to hGR binding to FITC-G2T in the control-IgG supernatants that is absent in the samples from FIGR supernatants. Western blotting of protein A agarose bead supernatants from these reactions confirmed that the hGR had been removed from the supernatant by the FIGR antibody but not by the control IgG (data not shown). Thus, the change is mP appears to be entirely due to binding of the FITC-G2T by the hGR.

Supplemental Figure Captions.

Figure S1. Effect of salt on hPR binding to FITC-G2T and to FITC-Random. hPR (64 nM) was incubated with 1 nM FITC-G2T (■) or 1 nM FITC-Random (▲) in FP buffer with increasing concentrations of salt (NaCl; shown on the x-axis), and FP was measured at equilibrium as described in Experimental Procedures. "Change in polarization (mP)" represents the difference between polarization values measured at each concentration of receptor and the polarization measured with no added receptor. The data are a representative experiment from two independent repeats. Duplicates were measured for each data point within this experiment, and the error bars shown are average error.

Figure S2. Under FP buffer conditions, there is no nonspecific binding of hPR to FITC-Random. Increasing amounts of hPR were incubated with 1 nM FITC-G2T (■) or FITC-Random (▲) in FP buffer, and FP was measured at equilibrium as described in Experimental Procedures. "Change in polarization (mP)" and error bars are as described in Figure 8 legend. The data are a representative experiment from two independent repeats. Three replicates were measured for each data point within each independent experiment.

Figure S3. Glutathione moderately enhances hPR binding to FITC-G2T. Increasing amounts of hPR were incubated with 1 nM FITC-G2T in FP buffer without (■) or with (▲) 1 mM GSH, and FP was measured at equilibrium as described in Experimental Procedures. "Change in polarization (mP)" and error bars are as described in the Figure 8 legend. Three replicates were measured for each data point.

Figure S4. Competition experiments to assess the specificity of hGR and hPR binding to FITC-G2T. Receptor concentrations that produce approximately 70% of maximal binding were incubated with 1 nM FITC-G2T and with the indicated fold-molar excesses of unlabeled G2T or nonspecific Random oligonucleotide in FP buffer (plus 1 mM GSH and 7.5 mM CHAPS for hGR only), and FP was measured at equilibrium as described in Experimental Procedures. N.C. = no competitor. Panel A: hGR; Panel B: hPR. Percent bound values on the

y-axes were calculated by dividing the measured "change in polarization" (see description of this calculation and error in the Figure 8 legend) by the "change in polarization" measured in samples without any added competitor. The error-propagated standard deviations were divided by each "change in polarization" absolute value to yield percentage errors, plotted here as error bars. Fold molar excess of unlabeled G2T or Random over FITC-G2T in each sample is indicated on the x-axes. Three replicates were measured for each data point.

Figure S5. Anti-hGR immunoprecipitation removes the species responsible for polarization increases upon FITC-G2T binding in the FP assay. The FIGR antibody (compared to a control IgG) was used to immunoprecipitate hGR as described in Experimental Procedures, and the resultant supernatants were incubated with 1 nM FITC-G2T in FP buffer in the presence of 1 mM GSH and 7.5 mM CHAPS (final). FP was measured at equilibrium as described in Experimental Procedures. "Change in polarization (mP)" is as described in Figure S1 legend. On the x-axis are indicated the type of antibody used in each sample's immunoprecipitation step (FIGR or control); 6 μL of each immunoprecipitation supernatant was added to each triplicate 40 uL FP reaction. The data represent the average of two independent experiments, ± average error.

Figures (Supplemental)

Fig S1

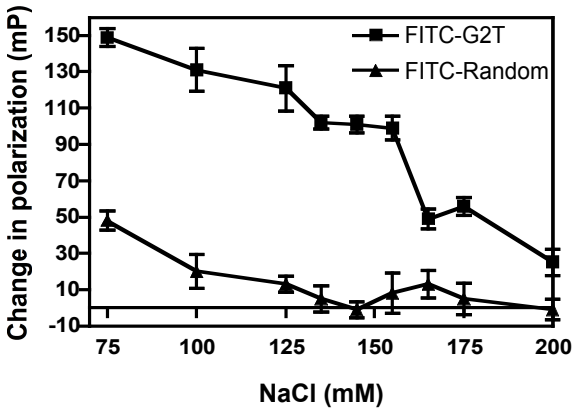


Fig S2

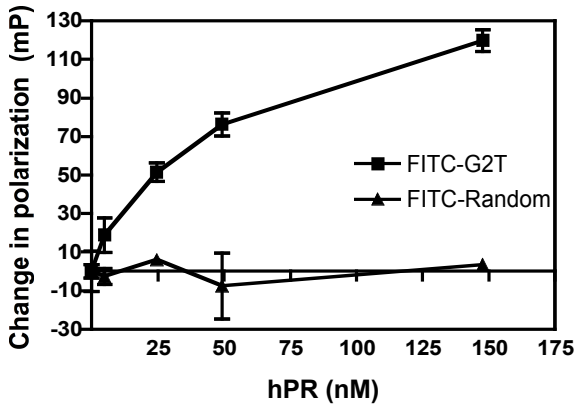

Change in polarization (mP)
130
110
90
70
50
30
10
-10
-30
25
50
75
100
125
150
175
hPR (nM)
FITC-G2T
FITC-Random

Fig S3

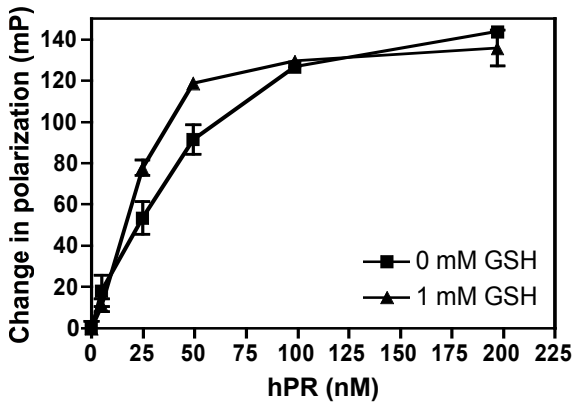
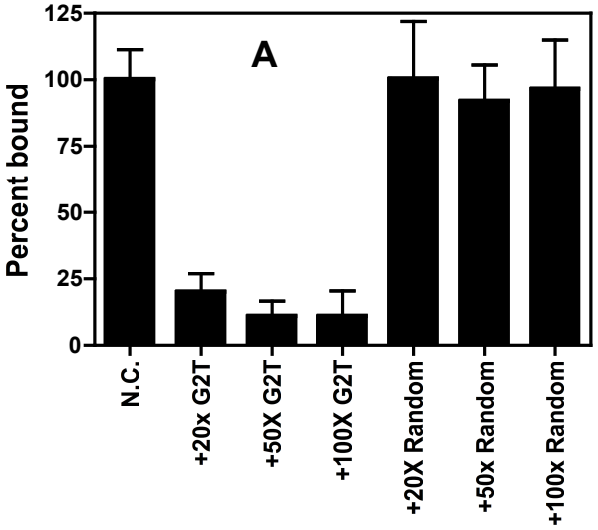

Change in polarization (mP)
140
120
100
80
60
40
20
0
0
25
50
75
100
125
150
175
200
225
hPR (nM)
0 mM GSH
1 mM GSH

Fig S4


A
Percent bound
125
100
75
50
25
0
N.C.
+20x G2T
+50X G2T
+100X G2T
+20X Random
+50x Random
+100x Random

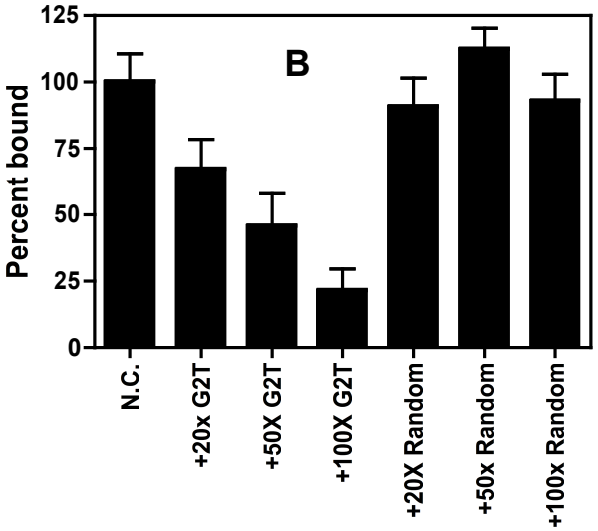

B
Percent bound
125
100
75
50
25
0
N.C.
+20x G2T
+50X G2T
+100X G2T
+20X Random
+50x Random
+100x Random

Fig S5