Analysis of Chicken Progesterone Receptor Function and Phosphorylation Using an Adenovirus-Mediated Procedure for High-Efficiency DNA Transfer†

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ABSTRACT: The expression of heterologous DNA in mammalian cells is crucial to understanding physiological functions or determining biological properties of encoded proteins. However, expression for biological assay or at levels sufficient for recovery and subsequent physical analysis has been limited by the poor efficiency, variability, or cost of current DNA transfer methodologies. We have modified a DNA transfer procedure which exploits the capacity of replication-deficient adenovirus to infect a wide range of cell types, carrying with it transiently associated DNA. We have established conditions for achieving 80% transfection of CV1 cells and have used this procedure for DNA transfer into several mammalian cell lines and primary cell cultures. We have shown that biologically active avian progesterone receptor may be readily detected, both immunologically and functionally, using less than 1 ng of progesterone receptor-encoding plasmid DNA per 2×10^5 cells. We previously reported the identification of four phosphorylation sites in chicken progesterone receptor using oviduct tissue minces labeled with [^{32}P]PO_4 under nonequilibrium conditions. We now find, using adenovirus-mediated infection and equilibrium labeling conditions, that the same sites are phosphorylated in receptor expressed in CV1 cells and report that there are no additional major phosphorylation sites in chicken progesterone receptor. The ease, efficiency, sensitivity, and wide applicability of this DNA transfer method should simplify current efforts to study heterologous protein expression in mammalian cells.

Heterologous expression of proteins is a key method for the analysis of the physiological function(s) of many proteins. However, many of the transient transfection procedures such as calcium phosphate coprecipitation or polybrene or DEAE-dextran-mediated transfection are relatively inefficient, resulting in a small percent of cells successfully transfected. A goal for DNA transfer and heterologous protein expression as well as for gene therapy is to achieve a greater population of transfected cells, with each cell expressing a low or physiologically relevant level of heterologous protein. Under these conditions, the endogenous levels of proteins or enzymes that interact with or modify the expressed protein should be sufficient to accurately measure the activity of the introduced protein. That transfected proteins are frequently expressed at levels that overwhelm the endogenous cellular components is suggested by studies showing the stimulation of the activity of human progesterone receptor in cells cotransfected with a plasmid that encodes the steroid receptor coactivator protein, SRC-1 (Onate et al., 1995).

Although some methods such as electroporation or the use of recombinant viruses to introduce DNA into cells are more efficient, neither technique is convenient for assays requiring differing levels of multiple plasmids in each sample. Liposome-mediated DNA transfer is fairly efficient under some circumstances but may be limited by cellular toxicity (Felgner et al., 1987; Malone et al., 1989), and the commercial reagent is quite expensive. A promising approach has been the use of replication-deficient adenoviruses to enhance DNA transfer (Curiel et al., 1991). Modified polylysine has been covalently bound to adenovirus using either an antibody (Curiel et al., 1992) or streptavidin−biotin (Wagner et al., 1992) bridge. DNA molecules will then ionically associate with the bound lysine, and as a result of adenovirus binding to its extracellular receptor and it undergoing endocytosis followed by endosome disruption, the linked heterologous DNA is released into the cytoplasm.

Our goal is to understand the role of phosphorylation in progesterone receptor function. Accordingly, we must express the receptor or mutants under conditions that will promote appropriate phosphorylation and that will allow us to detect changes in activity at physiological concentrations of receptor. Studies identifying phosphorylation sites of endogenous estrogen receptors expressed in MCF-7 cells (Arnold et al., 1994, 1995) have yielded sites different from those of estrogen receptors transiently overexpressed in COS cells (Ali et al., 1993; Le Goff et al., 1994) possibly because of overexpression in COS cells. We describe here a simplified method of adenovirus-mediated DNA delivery based on the method of Cristiano et al., (1993a) modified for use in transient transfections. This procedure takes advantage of two observations. Adenovirus receptors are essentially ubiquitously expressed, and endocytosis through the adenovirus receptor is sufficient for heterologous DNA transfer. In this procedure, polylysine is covalently bound to coat proteins of the adenovirus molecule using a water-soluble carbodiimide. Plasmid DNA then transiently associates with the bound polylysine, and this ionic interaction

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is sufficient to enable plasmid DNA to enter the cell with endocytosed virus particles.

We have used this adenoviral-mediated method of DNA delivery to study progesterone receptor function and phosphorylation in a heterologous cell line. We find that receptor expressed in this manner is functionally indistinguishable from receptor expressed through other conventional methods. We find that the progesterone receptor is phosphorylated in a hormone-dependent manner consistent with our observations in tissue oviduct minces. Using transfected CV1 cells uniformly labeled with [^{32}P]phosphate, we have now been able to determine that there are no additional major phosphorylation sites in chicken progesterone receptor.

EXPERIMENTAL PROCEDURES

Materials. 3-Hydroxytyramine (dopamine), 8-bromo-cAMP, poly(L-lysine) (molecular weight of approximately 25 000), chloramphenicol, and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) were from Sigma. DMEM for cell culture was from Gibco BRL Life Technologies. Fetal bovine serum was from Hyclone. Progesterone was from Steraloids; [^{3}H]progesterone (51 Ci/mmol) was from Amersham. Enhanced chemiluminescent (ECL) reagents for immunodetection were from Amersham. The progesterone receptor-specific monoclonal antibody PR22 (Sullivan et al., 1986) was kindly provided by Dr. David Toft (Mayo Clinic, Rochester, MN). Butyryl-coenzyme A was from Pharmacia, and [^{3}H]chloramphenicol (specific activity of 37 Ci/mmol) was from Dupont/New England Nuclear. The β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside) was from 5-Prime 3-Prime. Carrier-free [^{32}P]H_3PO_4 [285 Ci/(mg of phosphorus)] was from ICN. Tosylphenylalanyl chloromethyl ketone-treated trypsin was from Cooper Biomedicals. Tetramethylpentadecane (TMPD) and xylenes were from Fisher. HPLC reagents were from J. T. Baker Chemical Co.

Cell Culture and Adenovirus Infection. CV1 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Cells were plated into six-well cluster plates at a density of 2×10^5 cells per well approximately 24 h prior to infection. At the time of infection, the normal growth medium was removed and replaced with $^1/_2$ volume of DMEM without serum. Adenovirus−DNA−lysine complex was added to the cells, and cells were incubated for 2 h to allow virus infection. At this time, cells received an equal volume of medium supplemented with 10% charcoal-stripped fetal bovine serum. Hormones or other agents were added to the cells approximately 30 h after infection as indicated, and cells were harvested for analysis 15 to 18 h later, approximately 48 h after infection.

Plasmids. The plasmid CMV βGal contains the β-galactosidase gene under the transcriptional control of the cytomegalovirus (CMV) promoter and enhancer. The avian progesterone receptor A form is synthesized from the plasmid pADcPR$_A$ under the transcriptional control of the adenovirus major late promoter in p91023b. The hormonally responsive CAT reporter vector GRE$_2$E1bCAT (Allgood et al., 1993) contains two copies of the glucocorticoid/progesterone response element from the tyrosine aminotransferase gene fused to an oligonucleotide encoding the adenovirus E1b TATA sequence, followed by the bacterial chloramphenicol acetyltransferase (CAT) coding sequence.

Adenovirus Preparation. Replication-deficient adenovirus dl312, obtained from S. Woo (Baylor College of Medicine), was propagated in 293 cells as described (Cristiano et al., 1993b); 293 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. The virus was purified by double banding on cesium chloride gradients followed by dialysis against Hepes-buffered saline (HBS, 150 mM NaCl and 20 mM HEPES at pH 7.3) as described (Cristiano et al., 1993b). Virus titer was checked by mixing 25 μL of virus with 465 μL of phosphate-buffered saline and 10 μL of 5% SDS. The sample was vortexed for 2 min and centrifuged for 2 min to remove insoluble material and absorbance at 260 nM determined. One optical density (OD) unit equals approximately 1×10^{12} particles/mL. Virus to be used for DNA transfer was transferred to polystyrene tubes and immediately covalently modified with poly(L-lysine). For this, virus (1.4×10^{11} particles) was incubated with 16 μM poly(L-lysine) and 130 μM 1-ethyl-3-[3-(dimethylamino)-propyl)]carbodiimide (EDC) in a final volume of 4 mL for 4 h at 4 °C in the dark; unreacted reagents were removed by ultracentrifugation through cesium chloride. The virus was dialyzed overnight against sterile 2 M NaCl and 20 mM HEPES at pH 7.3 and 4 °C in the dark. Modified virus was then brought to 10% glycerol and stored at −80 °C. Adenovirus−DNA complexes were prepared by incubating lysine-modified adenovirus with plasmid DNA for 30 min at room temperature in the dark, followed by a 30 min incubation with polylysine at a molar concentration equivalent to 125 times the molar plasmid DNA concentration. This incubation was also at room temperature in the dark. Specific amounts of virus and plasmid DNA are defined in the figure legends. Adenovirus−lysine−DNA complexes were immediately added to cultured cells as described above.

Analysis of Progesterone Receptor Expression. After infection, cells were harvested by scraping in TEN buffer (40 mM Tris, 1 mM EDTA, and 150 mM NaCl) and resuspended in high-salt homogenization buffer [0.4 M NaCl, 50 mM KPO_4 (pH 7.4), 10 mM $NaMoO_4$, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, and 12 mM α-monothioglycerol (pH 7.0)] and lysed by three cycles of freezing and thawing. Cellular debris was removed by centrifugation at 14 000 rpm for 5 min at 4 °C and the protein concentration determined in the colorimetric Bradford analysis (Bradford, 1976). Aliquots containing equivalent amounts of protein were electrophoresed through 6.5% polyacrylamide−SDS gels and electrophoretically transferred to nitrocellulose by the method of Towbin (1979). Progesterone receptor levels were determined using the progesterone receptor-specific antibody PR22 in a chemiluminescent immunodetection system as described (Weigel et al., 1992).

Analysis of Progesterone Receptor-Mediated Gene Expression. Infected cells were harvested by scraping as above, resuspended in 0.25 M Tris (pH 7.4), and lysed by three cycles of freezing and thawing. Cellular debris was removed by centrifugation at 14 000 rpm for 5 min at room temperature. The protein concentration was determined in the colorimetric Bradford analysis (Bradford, 1976). CAT activity was determined in aliquots containing 5 or 10 μg of protein as described (Zhang et al., 1994a; Brian & Sheen, 1988). Briefly, aliquots were incubated with *n*-butyryl-coenzyme A (final concentration of 0.25 mg/mL) and [^{3}H]-chloramphenicol (28 mM, specific activity of 54 mCi/mmol) for 30 min at 37 °C. Butyrylated [^{3}H]chloramphenicol

reaction products were extracted with two volumes of tetramethylpentadecane/xylenes (2:1), and radioactivity was determined by scintillation counting.

Equilibrium Steroid Hormone Binding Analysis. CV1 cells were plated at a density of 3×10^6 cells per 150 mm dish and infected with adenovirus−DNA complex (20 μg of plasmid DNA per dish). Thirty-six hours after infection, cells were harvested by scraping into phosphate-buffered saline (PBS), resuspended in 50 mM Tris, 1 mM EDTA, 12 mM α-monothioglycerol, and 20 mM sodium molybdate at pH 7.5, and manually homogenized in a ground-glass homogenizer at 4 °C. Cytosolic extracts were prepared by ultracentrifugation of the homogenate at 100 000 rpm for 10 min at 4 °C. Aliquots of cytosol containing equal amounts of protein were removed to prechilled tubes containing [^{3}H]progesterone at concentrations ranging from 0.05 to 10 nM with or without an excess of radioinert progesterone. After incubation on ice for 2 h, an aliquot was removed for quantitation of the total steroid concentration; the concentration of bound steroid was determined by hydroxylapatite adsorption as described (Clark & Peck, 1977). Data were analyzed by the method of Scatchard (1949) to determine the dissociation constant.

In Situ Analysis of β-Galactosidase Activity. The growth medium was removed from infected cells. Each well was washed twice with ice-cold phosphate-buffered saline (PBS), incubated for 5 min at room temperature with ice-cold 0.5% glutaraldehyde in PBS, and then washed twice more with ice-cold PBS. The β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside) was then added as described (MacGregor et al., 1987) and incubated at 37 °C. β-Galactosidase activity was visually apparent within 1 h and incubation was continued for an additional 12−24 h. The staining solution was then aspirated, and cells were rinsed twice with PBS and photographed.

In Vivo Phosphorylation of Receptor and Phosphopeptide Analysis. CV1 cells, plated at a density of 3×10^6 cells per 150 mm dish, were infected with adenovirus (750 virions per cell) incubated with 35 μg of cPR_A expression vector and subsequently with a 75-fold molar excess of poly(L-lysine). Thirty-three hours after infection, cells were rinsed with medium without phosphate and incubated for 1 h in phosphate-free medium. Cell growth medium was replaced with 10 mL of phosphate-free medium supplemented with 1% charcoal-stripped, dialyzed phosphate-free fetal bovine serum containing [^{32}P]orthophosphate (5 mCi/dish). After 2 h of incubation, progesterone (100 nM) was added or not and incubation continued for 15 h. Cells were harvested, resuspended in a minimal volume of homogenization buffer (50 mM KPO_4, 20 mM $NaMoO_4$, 50 mM NaF, 2 mM EDTA, and 2 mM EGTA) containing 0.4 M NaCl and 1% Triton X-100, and lysed by vortexing. A high-speed extract was prepared by centrifugation at 100 000 rpm for 12 min at 4 °C, and the extract was diluted with 4 volumes of homogenization buffer without Triton X-100. The cytosol was chromatographed over a PR22 immunoaffinity column as described (Poletti et al., 1993) and receptor eluted with 1 M acetic acid. After electrophoresis through SDS−polyacrylamide gels and subsequent autoradiography, the region of the gel containing the ^{32}P-labeled receptor was excised, crushed by manual homogenization in a glass-on-glass homogenizer in 50 mM ammonium bicarbonate, brought to 0.1% SDS and 5% β-mercaptoethanol, and incubated with shaking at 37 °C for 12 h. The acrylamide fines were collected by centrifugation at 14 000 rpm for 5 min at room temperature, and protein in the supernatant was precipitated by incubation with trichloroacetic acid (TCA, final concentration of 20%) for 1 h on ice, followed by centrifugation at 14 000 rpm for 30 min at 4 °C. Recovered receptor was dissolved in 200 μL of 50 mM ammonium bicarbonate, TPCK-treated trypsin added to a concentration of 20 μg per 200 μL, and the digestion allowed to proceed at 37 °C for approximately 12 h. Tryptic phosphopeptides were then analyzed by reverse-phase HPLC as described (Denner et al., 1990a). Briefly, phosphopeptides were applied to a Vydac C_{18} reverse-phase column in 0.1% trifluoroacetic acid in water (eluant A) at a flow rate of 1 mL/min. Peptides were eluted with a linear gradient from 0 to 45% acetonitrile in eluant A over 90 min. ^{32}P-labeled peptides were identified on-line using a radioactivity flow detector.

RESULTS

To determine the ratio of adenovirus particles per cell which results in maximal heterologous gene expression in our target cell line, lysine-modified adenovirus was incubated with the plasmid CMVβGal and CV1 cells were infected with increasing amounts of the virus−DNA complex. After 18 h, the level of β-galactosidase activity was determined by *in situ* analysis. Results of this analysis are presented in Figure 1. From panels E and F, it is apparent that conditions were established which result in very efficient delivery of the plasmid DNA. At virus:cell ratios of 500:1 and 750:1, approximately 80% of the cells exhibit blue reaction product, indicative of expression from the heterologous plasmid. In this same experiment, companion cells were transfected with the same plasmid DNA through a standard procedure which utilizes the cationic reagent polybrene. This procedure has been used previously successfully to transiently introduce plasmid DNA (Zhang et al., 1994a), but the transfection efficiency is quite low, as seen in panel A of Figure 1.

To establish conditions for expression of biologically active progesterone receptor, lysine-modified adenovirus was incubated with two expression vectors, one encoding the progesterone receptor and one containing the hormonally responsive CAT reporter vector $GRE_2E1bCAT$. For each well of a six-well cluster plate, 0.1 ng of receptor plasmid, 250 ng of CAT reporter, and the indicated number of virus particles per cell were used to infect CV1 cells which had been plated at a density of 2×10^5 cells per well. Twenty-four hours after infection, cells were treated with 100 nM progesterone or not and allowed to incubate for an additional 15 h. CAT activity was then determined using 10 μg of protein extract in a 60 min reaction.

As shown in Figure 2, cells grown in the absence of hormone show little CAT activity and progesterone treatment results in an increase in the level of CAT activity. We have shown previously that this effect of progesterone is mediated through the progesterone receptor, as no CAT activity is detected in cells treated with progesterone following transfection with CAT reporter only. The CAT activity increased as the virus:cell ratio increased to a maximum at 750:1. For this batch of virus, 1000:1 gave poorer results. This is consistent with the observation that infection at higher virus concentrations results in a greater proportion of transduced cells (Figure 1). For comparison, we transfected companion

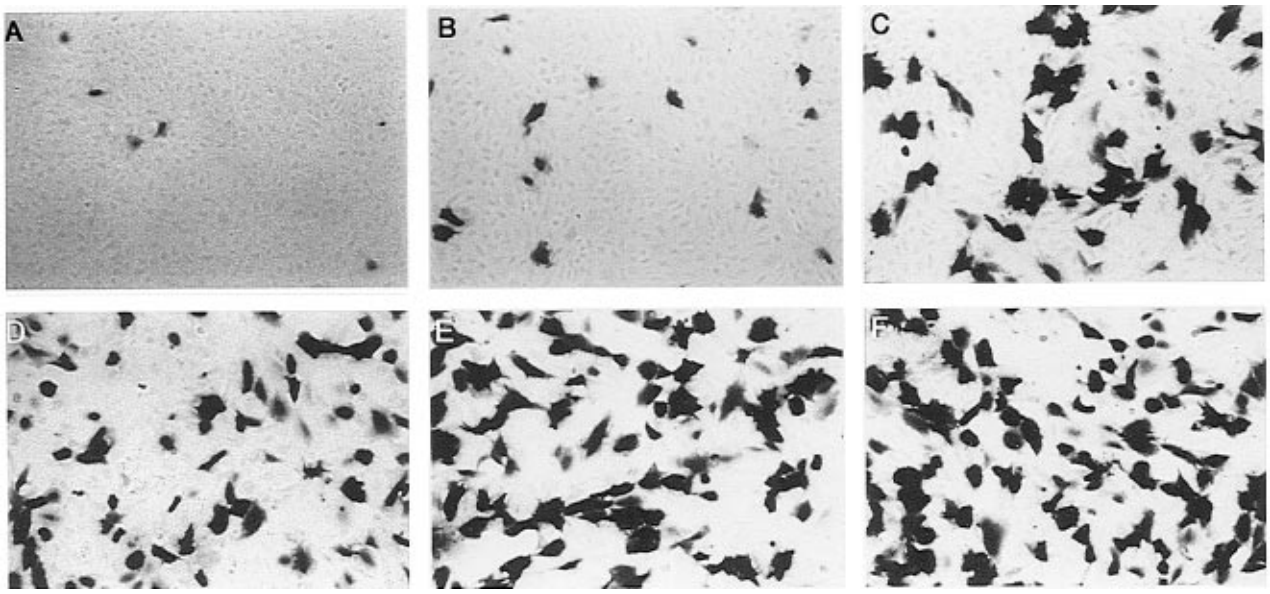

FIGURE 1: Efficiency of conventional transient transfection compared with adenovirus-mediated DNA transfer. The plasmid CMV βGal, in which β- galactosidase expression is under transcriptional control of the strong constitutive cytomegalovirus (CMV) promoter, was introduced into CV1 cells. In panel A, cells were transfected using the cationic agent polybrene (hexadimethrine bromide), a conventional transient transfection agent as described previously (Zhang et al., 1994a). In panels B−F, cells were infected with adenovirus−CMV βGal complex at adenovirus:cell ratios of (B) 10:1, (C) 100:1, (D) 300:1, (E) 500:1, and (F) 750:1. Eighteen hours after transfection or infection, cells were fixed and β-galactosidase activity detected by incubation *in situ* with the substrate x-gal (5-bromo-4-chloro-3-indoyl β-D-galactopyranoside).

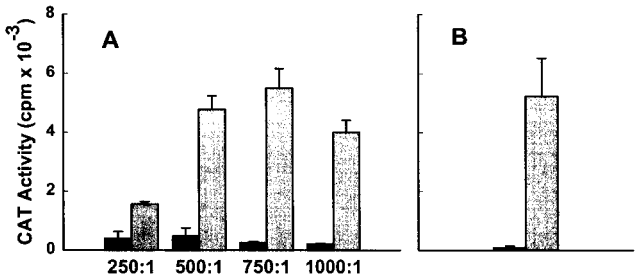

FIGURE 2: Comparison of transcriptional activation of progesterone receptor produced through adenovirus-mediated DNA transfer and conventional transient transfection. CV1 cells (2×10^5) were infected with adenovirus coupled with 0.1 ng of cPR_A expression vector and 250 ng of $GRE_2E1bCAT$, a progesterone-responsive CAT reporter vector. In panel A, cells were infected at the indicated virus:cell ratio; in panel B, CV1 cells (1×10^6 per 100 mm dish) were transfected with 5 μg of cPR_A expression vector and 5 μg of $GRE_2E1bCAT$ using the cationic agent polybrene as previously described (Zhang et al., 1994a). Twenty-four hours after transfection or infection, cells were treated with progesterone (Prog, 100 nM) or not (Con) for an additional 15 h and harvested, and CAT activity was determined. For cells infected via adenovirus (panel A), the CAT assay was performed using 10 μg of cell extract in a 60 min enzymatic assay. For cells transfected via polybrene (panel B), the CAT assay was performed using 50 μg of cell extract in an 18 h enzymatic reaction. Samples were done in triplicate, and data are presented ±SEM.

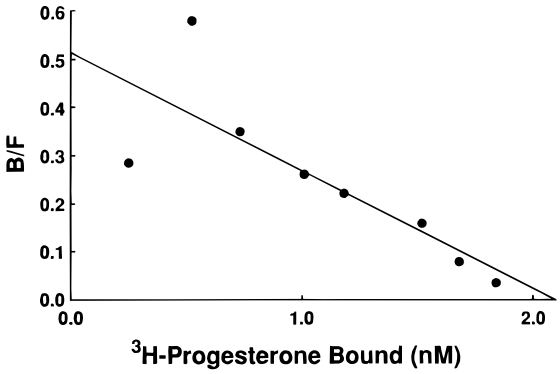

FIGURE 3: Equilibrium hormone binding analysis. CV1 cells were plated and infected (750 virions per cell) as described in Experimental Procedures. Thirty-six hours after infection, cells were harvested and cytosolic extracts prepared, and [^{3}H]progesterone binding was measured. Data were analyzed by the method of Scatchard (1949) to determine hormone binding affinity.

cells using the conventional polycation polybrene. For this, cells were plated at a density of 1×10^6 cells per 100 mm dish and transfected with 5 μg of receptor expression vector and 5 μg of CAT reporter. These are conditions established previously within this laboratory that reproducibly yield progesterone receptor-mediated CAT reporter activity (Zhang et al., 1994a). The level of CAT activity in 50 μg of cellular extract from cells transfected via the polybrene method analyzed in an 18 h reaction (Figure 2, panel B) was comparable to the level obtained with 10 μg of cellular extract from cells infected with adenovirus−DNA complex in a 60 min reaction (Figure 2, panel A).

[^{3}H]Progesterone binding assays demonstrated that progesterone receptor produced through this method of DNA delivery binds progesterone with an affinity of approximately 4 nM, as shown in Figure 3, consistent with the affinity of endogenous chicken oviduct progesterone receptor for progesterone (Maggi et al., 1984).

A particularly important feature of any DNA delivery method is that the biological response or level of heterologous protein increases with increasing DNA concentrations. To determine if this method of adenovirus-mediated DNA delivery would be responsive to changes in DNA concentration, CV1 cells were infected with adenovirus carrying 200 ng of CAT reporter and increasing amounts of receptor expression vector, ranging from 0.1 to 2 ng. After exposure to progesterone or not, cells were harvested and the level of progesterone-induced CAT activity was determined. Results of this analysis are presented in Figure 4 and show that the level of hormone-induced CAT gene expression increases with increasing amounts of up to 1 ng of receptor expression

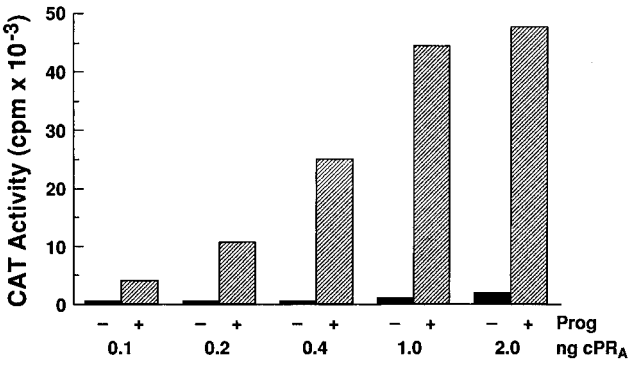

FIGURE 4: Effect of progesterone receptor expression vector concentration on the level of progesterone-induced gene expression. CV1 cells, plated at a density of 2×10^5 cells per well, were infected with adenovirus (750 virions per cell) carrying 200 ng of $GRE_2E1bCAT$ and various amounts of cPR_A expression vector, ranging from 0.1 to 2.0 ng as indicated. Twenty-four hours after infection, cells were treated with progesterone (Prog, 100 nM) or not (Con) and harvested 15 h subsequently. CAT activity was determined using 5 μg of cell extract in a 30 min enzymatic reaction.

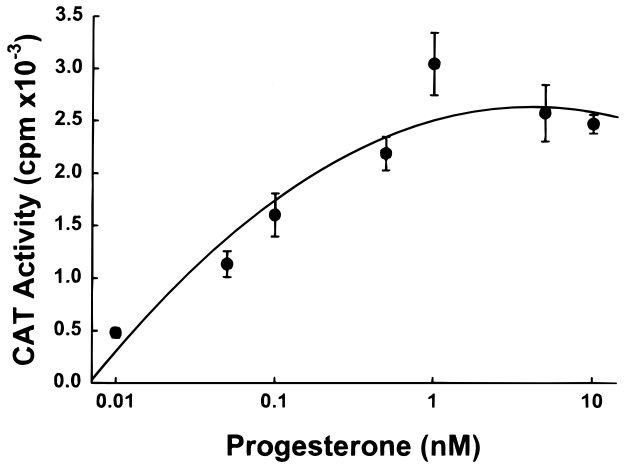

FIGURE 5: Effect of progesterone concentration on the level of receptor-mediated gene expression. CV1 cells, plated at a density of 2×10^5 cells per well, were infected with adenovirus (750 virions per cell) carrying 0.1 ng of cPR_A expression vector and 250 ng of $GRE_2E1bCAT$. Twenty-four hours after infection, cells were treated with progesterone (Prog) at concentrations ranging from 0.01 to 100 nM as indicated, or not (con), and harvested 15 h later. CAT activity was determined using 10 μg of cell extract in a 60 min enzymatic reaction. Samples were done in triplicate, and data are expressed ±SEM.

vector; basal expression in the absence of progesterone changes very little in response to increased receptor expression vector amount.

The hormone dependence of progesterone receptor activation expressed through adenoviral-mediated DNA delivery is shown in Figure 5 and demonstrates that receptor-mediated gene expression occurs in a progesterone dose-dependent manner in the presence of low steroid concentrations, reaching a relative plateau as the concentration is raised. From this representative experiment and others not shown, half-maximal gene expression is detected at progesterone concentrations around 0.1 nM, similar to the results obtained for chicken progesterone receptor in conventional transient transfections (Bai et al., 1994).

To determine the level of expression of cPRa in the transfected cells, varying amounts of receptor plasmid were coupled to adenovirus and the transfected cells harvested, receptor extracted as described in Experimental Procedures,

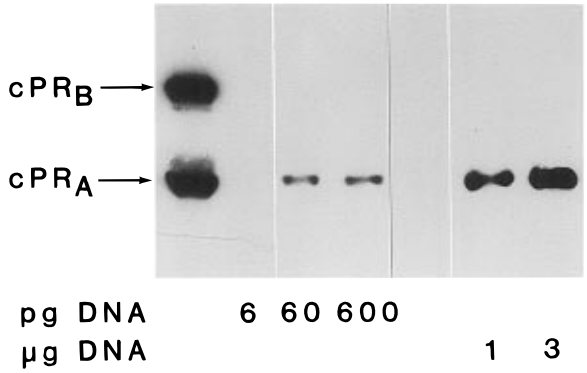

FIGURE 6: Receptor DNA concentrations required for minimal and maximal levels of progesterone receptor expression. CV1 cells, plated into individual wells of six-well cluster plates at a density of 2×10^5 cells per well, were infected with adenovirus (750 virions per cell) carrying cPR_A expression vector amounts ranging from 6 pg to 3 μg as indicated. After 36 h, cells were harvested and high-salt extracts were prepared. Aliquots of 10 μg from each condition were electrophoresed through SDS−polyacrylamide gels and transferred to nitrocellulose, and levels of cPR_A and quantitated by immunoanalysis using the progesterone receptor-specific antibody PR22. This antibody recognizes both A and B forms of the progesterone receptor. In the left-most lane, 30 μg of chicken oviduct cytosolic protein was simultaneously processed. The A and B forms of progesterone receptor present in the oviduct cytosol sample are indicated. From [^{3}H]progesterone binding analysis, 30 μg of oviduct cytosol contains approximately 3 ng of receptor, 1.5 ng of each form, A and B.

and analyzed by SDS gel electrophoresis followed by Western blotting with PR22, an antibody which specifically recognizes the chicken progesterone receptor. The results of these studies are presented in Figure 6. With as little as 60 pg of plasmid DNA per 2×10^5 cells, immunologically detectable receptor was clearly apparent. In an effort to maximize expression, larger amounts of plasmid DNA were incubated with virus. We found that 3 μg of plasmid DNA per 2×10^5 cells resulted in the highest level of receptor expression. Use of higher levels of plasmid DNA and correspondingly higher levels of polylysine was deleterious to the cells.

The progesterone receptor is a phosphoprotein, and the state of phosphorylation has been shown to increase in response to progesterone treatment (Denner et al., 1987, 1990a; Dougherty et al., 1982). In addition, the receptor can be transcriptionally activated in transiently transfected cells in response to treatment with agents that elevate intracellular protein kinase activity levels, such as 8-bromo-cAMP (Denner et al., 1990b) and growth factors (Zhang et al., 1994a). Additionally, agents which inhibit protein phosphatase activity, such as okadaic acid and sodium vanadate (Zhang et al., 1994a), also result in transcriptionally active receptor. Lastly, the neurotransmitter dopamine has been shown to activate the receptor (Power et al., 1991b); although the mechanism through which dopamine acts is unclear, it is known that dopamine can influence the activities of several protein kinases and phosphatases (Felder et al., 1984, 1989; Hemmings et al., 1984). Previous studies were conducted under conditions in which receptor expression per cell was supraphysiological. To determine whether progesterone receptor can be activated by ligand-independent pathways under conditions where receptor levels are physiological or even lower, cells were infected with adenovirus−DNA complexes containing progesterone receptor and

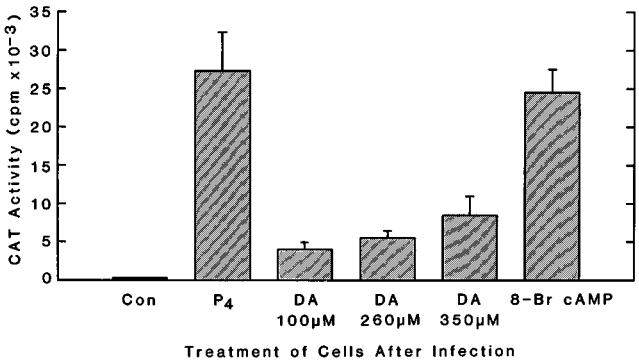

FIGURE 7: Progesterone-dependent and -independent transcriptional activation of progesterone receptor. CV1 cells, plated into individual wells of six-well cluster plates at a density of 2×10^5 cells per well, were infected with adenovirus (750 virions per cell) carrying 0.06 ng of cPR_A expression vector and 250 ng of $GRE_2E1bCAT$, a progesterone-responsive CAT reporter vector. Twenty-four hours after infection, cells were treated with progesterone (Prog, 100 nM), dopamine (DA) at the concentrations indicated, or 8-bromo-cAMP (8-Br-cAMP, 1 mM) or not (Con). After 15 h of treatment, cells were harvested and levels of progesterone receptor-mediated CAT gene expression quantitated with 10 μg of cytosolic protein in a 30 min assay.

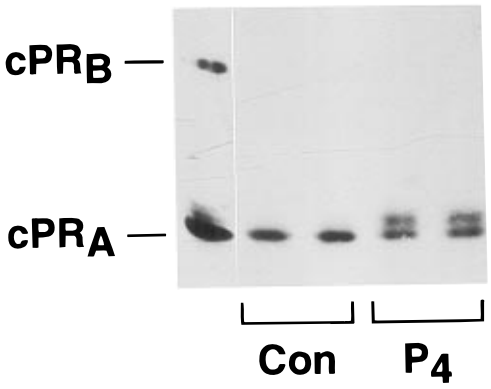

FIGURE 8: Effect of progesterone treatment on electrophoretic mobility of progesterone receptor. CV1 cells, plated into individual wells of six-well cluster plates at a density of 2×10^5 cells per well, were infected with adenovirus (750 virions per cell) carrying 0.6 ng of cPR_A expression vector. Twenty-four hours after infection, cells were treated for 15 h with 100 nM progesterone (Prog) or not (CON). High-salt extracts were prepared, and protein samples of 10 μg were electrophoresed through SDS−polyacrylamide gels, transferred to nitrocellulose, and progesterone receptor localized by immunoreaction with the progesterone receptor-specific antibody PR22. Chicken oviduct cytosol (30 μg of protein) processed simultaneously is shown in the left-most lane. Electrophoretic mobilities of the A and B forms of the progesterone receptor are indicated.

reporter vector as described above. Twenty-four hours after infection, cells were treated with progesterone, dopamine at the concentrations indicated, or 8-bromo-cAMP as indicated and allowed to incubate for an additional 15 h. Results of CAT assays, shown in Figure 7, demonstrate that these agents act to increase the level of receptor-mediated CAT gene expression in a manner consistent with that previously demonstrated. This suggests that the receptor protein produced as a result of adenovirus-mediated DNA delivery is indistinguishable from that expressed through the conventional DNA transfer methods with respect to susceptibility to activation by signaling pathways active in the absence of cognate hormone and that ligand-independent activation is not a result of overexpression of receptor.

The hormone-dependent increase in receptor phosphorylation is accompanied by a decreased mobility on SDS gels (Denner et al., 1989). Treatment with phosphatase prior to electrophoresis eliminates the change in mobility, suggesting that the decrease is the result of progesterone-induced phosphorylation (Denner et al., 1990a). As a first test of whether phosphorylation of receptor expressed in CV1 cells is similar to phosphorylation of endogenous chicken progesterone receptor, CV1 cells were exposed to adenovirus−DNA complexes containing progesterone receptor expression vector. Cells were treated with 100 nM progesterone for 15 h prior to collection, and receptor was analyzed with respect to electrophoretic mobility. Results from this analysis, shown in Figure 8, demonstrate that receptor isolated from hormone-free cells electrophoreses as a single species with a molecular weight of 79 000 and comigrates with the authentic progesterone receptor A form isolated from chicken oviduct, shown in the left-most lane. We found that, in response to progesterone treatment, a portion of the receptor exhibited a decrease in electrophoretic mobility. This upshift is consistent with known effects of progesterone treatment on endogenous receptor (Denner et al., 1990a).

Previous work from this group has demonstrated that the progesterone receptor in chicken oviduct is phosphorylated to a low extent on two sites, serines 211 and 260, in the absence of hormone and undergoes additional phosphorylation of these two sites as well as two additional sites, serines 367 and 530, in response to progesterone treatment (Denner et al., 1990a; Poletti & Weigel, 1993). However, these studies were done under nonequilibrium labeling conditions in oviduct tissue minces, and it was possible that we had missed constitutive sites that turn over slowly or sites that are phosphorylated slowly in response to treatment with progesterone. Therefore, to determine if progesterone receptor expressed in CV1 cells through adenovirus-mediated DNA delivery would exhibit this same pattern of phosphorylation or would reveal novel sites, CV1 cells were infected with adenovirus coupled with progesterone receptor expression vector and treated with progesterone or not in the presence of [^{32}P]orthophosphate. Receptor was isolated by affinity chromatography using the antireceptor antibody PR22. The HPLC elution profiles and electrophoretic mobility patterns of tryptic phosphopeptides from both control- and progesterone-treated samples were compared with profiles of receptor purified identically from chicken oviduct tissue minces as described (Denner et al., 1990a). We found that the HPLC elution profiles, shown in Figure 9, match the elution profiles of receptor from chicken oviduct (Denner et al., 1990a) and receptor expressed and phosphorylated in yeast (Poletti et al., 1993). We observed low but detectable phosphorylation of the receptor from cells not treated with progesterone, shown in the lower panel of Figure 9. Subsequent analysis of these phosphopeptides by acrylamide electrophoresis (not shown) confirmed that the peptide containing phosphoserine 260 is present in the phosphopeptide broadly eluting at 55 min (peak 2) and the peptide containing phosphoserine 211 is present in the peak eluting at 75 min (peak 1). In contrast, progesterone treatment resulted in significantly enhanced phosphorylation of the receptor, and the elution profile of tryptic peptides of this receptor is shown in the upper panel of Figure 9. As seen with receptor from control-treated cells, we found that phosphoserine 260 (site 2) in the phosphopeptide eluting at

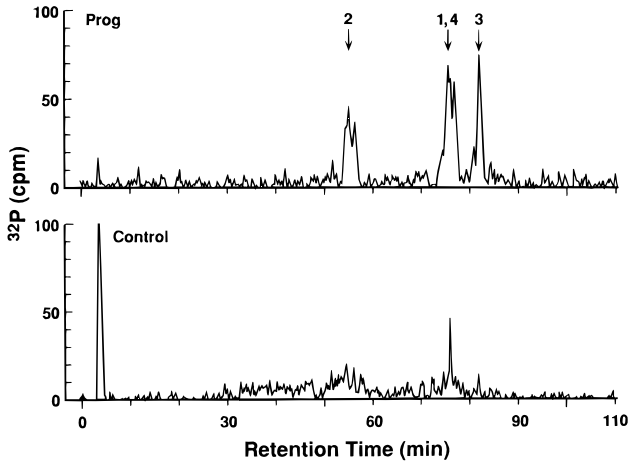

FIGURE 9: HPLC elution profiles of progesterone receptor tryptic phosphopeptides. CV1 cells were infected with adenovirus carrying cPR_A expression vector. Thirty-six hours after infection, cells were treated with progesterone (Prog, 100 nM) or not (Control) for 15 h in phosphate-free medium supplemented with carrier-free [^{32}P]-orthophosphate. Receptor was isolated and digested with trypsin, and phosphopeptides were resolved by reverse-phase HPLC and identified by radioactivity detection. The elution positions for authentic site 1 (Ser211), site 2 (Ser260), site 3 (Ser530), and site 4 (Ser367) peptides are indicated.

55 min (peak 2) and phosphoserine 211 (site 1) are present in the material eluting at 75 min (peak 1). In contrast to the receptor from control-treated cells, we observed a second phosphopeptide, containing phosphoserine 367 (site 4), coeluting with the 211-containing peptide at 75 min, and we found the phosphopeptide containing serine 530 (site 3) eluting at 82 min, giving rise to peak 3. Thus, we find that chicken progesterone receptor heterologously expressed in CV1 cells, treated with and without progesterone, is phosphorylated in a manner consistent with endogenous receptor from chicken oviduct exposed to the same progesterone conditions and that there are no additional major phosphorylation sites in cPR.

DISCUSSION

A major problem in studying heterologously expressed steroid receptors as well as other proteins in transiently transfected cells has been the inefficiency of most transfection procedures. Low-level expression permits analysis of functional consequences of interactions with endogenous factors that may bind to the same promoter, act as coactivators or corepressors, or post-translationally modify the protein and provides the conditions for detecting changes in functions such as affinity for DNA. Our interest in steroid receptor phosphorylation led us to adapt an adenovirus-mediated DNA delivery system, described for gene therapy (Cristiano et al., 1993a), for use as a general transfection procedure for a wide variety of cells. Using a reporter with a minimal promoter, GRE_2E1bCAT, that responds only to steroid receptors that bind to a GRE/PRE, we have found that this technique produces very little activity in the absence of activator (the [^{3}H]chloramphenicol reagent blank contributes 250 cpm of the background, and the remaining is contributed by background activity) and that we can achieve activities of 30−50 000 cpm in the presence of activators (see Figure 4) providing a high-fold activation and a wide activity range for studying receptor function. This contrasts with the polybrene method used previously, which has a limited range of activation for chicken progesterone receptor (Zhang et al., 1994a).

Furthermore, we can measure receptor expression levels in the same samples. This is particularly important in comparing the activities of two receptors which may exhibit subtle changes in activity due to altered phosphorylation or single amino acid mutations or whose stability or expression may change as a result of mutation or post-translational modification. From [^{3}H]progesterone-binding studies, 30 μg of chicken oviduct cytosol (Figure 6, left-most lane) contains approximately 3 ng of progesterone receptor, with 1.5 ng each of form A and B. Thus, the receptor A form comprises 0.005% of the total high-salt-extractable protein. On the basis of comparison with endogenous receptor in chicken oviduct, 10 μg of high-salt-extractable protein from CV1 cells resulted in the production of approximately 1 ng of immunologically detectable receptor protein. This level of receptor, 1 ng per 10 μg of protein, represents approximately 0.01% of the total high-salt-extractable protein and corresponds to approximately 40 000 receptor molecules per cell or twice the level of the receptor A form expressed endogenously in chicken oviduct. Lower levels of transfected receptor DNA yield lower levels of expressed receptor (Figure 6). On the basis of the efficiency of transfection and the expression levels of the receptor, we find that we can readily assay receptor activity at subphysiological levels of receptor expression.

Other adenovirus-mediated methods have been described (Curiel et al., 1992; Wagner et al., 1992) which likely would achieve the same goals, but the procedures for preparing the virus are somewhat more complicated. Although there is some effort involved in preparing the coupled virus (see Experimental Procedures), it can be stored at −70 °C for at least 3 months without loss of activity and thawed as needed. Typically, one batch of virus prepared from 25 T150 flasks of 293 cells is sufficient to transfect 5000 wells of CV1 cells under our conditions at a reagent cost of about $300, or less than $0.10/well. The decrease in the amount of DNA needed for transfections more than compensates for the labor involved in preparing the virus and also reduces the overall cost of the assay. We estimate that the assay is 25−100-fold more sensitive than conventional transient transfections; 10 μg of protein extract from adenovirus-infected cells incubated for 60 min produces as much acylated chloramphenicol as does 50 μg of extract from conventionally transfected cells incubated overnight under conditions where the assay is linear for at least 5 h. When higher levels of DNA are transfected by adenovirus-mediated transfection, the differences are even greater (Figure 4). The ability to use fewer cells not only results in substantial financial savings but also allows studies of primary cells which are difficult to obtain in large quantities.

Although this report has focused on the characteristics of cPRa expressed in CV1 fibroblast cells, we have found that the adenovirus works about as well in HeLa cells which are an epithelial cell line and in COS cells which can be used to produce higher levels of protein (data not shown). To optimize expression in a new cell type or to characterize a new batch of virus, we first determine the best virus:cell ratio to achieve optimal infection using CMV β-Gal and then titrate the amount of receptor and reporter plasmids to optimize the assay. Cells that are less readily infected, such as the PC3 prostate cancer cell line (Nazareth & Weigel,

1996), may require 2−3-fold more virus and 5−10-fold more DNA. While the efficiency of transfection may vary from cell line to cell line, to our knowledge, in all of the cell lines and primary cultures, including granulosa cells and sertoli cells, that we or others have tested using our virus, the efficiency of transfection is much better than that of previously used methods.

The functional properties of receptor expressed through adenovirus-mediated transfection, including hormone binding and transcriptional activation, are indistinguishable from those reported previously (Bai et al., 1994). The finding that the receptor activation curve does not match the hormone binding curve has been reported previously (Bai et al., 1994) and is due to phosphorylation of Ser530. Mutation of this site to Ala causes the two curves to match more closely (Bai et al., 1994). Studies to identify phosphorylation sites in steroid receptors have in a few cases utilized endogenous receptors (Denner et al., 1990a; Poletti & Weigel, 1993; Arnold et al., 1994; Zhang et al., 1994b, 1995) but in a number of cases have utilized receptors overexpressed in heterologous cells (Bodwell et al., 1991; Ali et al., 1993; Le Goff et al., 1994). These studies have led to conflicting conclusions. For example, both chicken and human progesterone receptor expressed in endogenous cells exhibit hormone-dependent phosphorylation of new sites (Denner et al., 1990a; Zhang et al., 1995), whereas rabbit progesterone receptor expressed by transient transfection of heterologous cells does not (Chauchereau et al., 1994). Whether this is a true species difference or is a result of the techniques used is not clear. Arnold et al., using endogenous human estrogen receptor from MCF-7 cells, have reported that Ser167 is the major hormone-dependent phosphorylation site (Arnold et al., 1994) and that Tyr537 (Arnold et al., 1995) is also phosphorylated. In contrast, both LeGoff et al. (1994) and Ali et al. (1993), using heterologously expressed human estrogen receptor, identified Ser118 as the major phosphorylation site and failed to detect phosphorylation of Ser167. The techniques used to identify sites (direct isolation of peptides versus deletion analysis) differed markedly, but the reasons for these discrepancies are not clear. Using the adenovirus-mediated transfection procedure which allows us to analyze receptor expressed at physiological levels, we found the same hormone dependence of phosphorylation detected in oviduct tissue minces. Moreover, the uniform labeling achieved in these studies leads us to conclude that there are no additional major phosphorylation sites in the chicken progesterone receptor. Hence, under these conditions, the phosphorylation matches endogenous phosphorylation, and this technique is suitable for evaluating the role of phosphorylation in receptor function.

The finding that some, but not all, of the steroid receptors can be activated in a ligand-independent manner raises the question of the role of these pathways in receptor function *in vivo*. Most of these studies have been done in transiently transfected cells where expression of receptor levels may be quite high (Denner et al., 1990b; Power et al., 1991a,b; Smith et al., 1993; Ignar-Trowbridge et al., 1993). We show here that these alternate pathways also function when the chicken progesterone receptor is expressed at physiological levels. These data are consistent with the findings using whole animals which show that the estrogen receptor can be activated by epidermal growth factor (Ignar-Trowbridge et al., 1992) and that the progesterone receptor-dependent lordosis response can be elicited by dopamine (Mani et al., 1994).

In summary, the ease, efficiency, and cost with which heterologous plasmid DNA can be transferred using the procedure described here should provide a significant improvement in the ability to study the biological functions and properties of proteins which must be heterologously expressed. In addition, the ubiquitous distribution of cell surface adenovirus receptors underscores the applicability of this method of DNA delivery to many cell types.

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