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Different Populations of Progesterone Receptor–steroid Complexes in Binding to Specific DNA Sequences: Effects of Salts on Kinetics and Specificity

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We previously reported evidence for two subpopulations of several classes of steroid receptors that could be distinguished by their requirement of a low molecular weight factor (M_r=700–3000 Da) for binding to nonspecific, calf thymus DNA–cellulose [Cavanaugh, A. H. and Simons Jr., S. S., Journal of Steroid Biochemistry and Molecular Biology, 48, 433–446 (1994)]. This factor appeared to be enriched in $(NH_4)_2SO_4$ precipitates of nuclear extracts. Using human progesterone receptors (PRs) and biologically active DNA sequences in a modified avidin/biotin-coupled DNA (ABCD) binding assay, we now report a factor-mediated increase in PR binding to specific DNA sites that was indistinguishable from that seen with nonspecific sites. The main advantages of this modified assay are that both kinetic and equilibrium binding of receptor-steroid complexes to DNA can be directly monitored in solution. The ability of either Sephadex G-50 chromatography or sodium arsenite to prevent that binding which is increased by added factor supported the existence of PR subpopulations that are independent of the acceptor DNA sequence. The factor was found, surprisingly, to be low concentrations (≥5 mM) of $(NH_4)_2SO_4$, which anomalously is partially excluded from Sephadex G-10 columns, and can be mimicked by some salts but not sodium arsenite. Kinetic analyses demonstrated that the mechanism of action of salt was to accelerate the rate of binding of PR. Salt also had a much greater effect on the nonspecific binding of PR, such that the ratio of specific to nonspecific DNA binding was greatest at elevated salt concentrations (~75 mM) that afforded submaximal levels of PR binding to specific DNA sites. Further analysis of the DNA-bound receptors revealed that the smaller, A-form of PR is preferentially bound to specific DNA sequences both in the presence and in the absence of various salt concentrations. Thus, the differences in DNA binding of PR ± salt do not correlate with the preferential binding of A or B isoform. The unequal behavior of PR subpopulations and/or isoforms for binding to specific DNA sequences offers added mechanisms for selective transcriptional regulation of genes in intact cells. Published by Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

The binding of receptor-steroid complexes to specific hormone responsive elements (HREs) in the DNA of regulated genes is an obligatory step in the control of gene transcription by steroid hormones. The HREs for the classical receptors of androgen, estrogen, glucocorticoid, mineralocorticoid and progestin steroids have been shown to be a 15 bp sequence containing two palindromic 6 bp sequences that are separated by

3 nucleotides. These HREs have the properties of enhancers since they are capable of conferring steroid inducibility to foreign genes (reviewed in [1, 2]). The first HRE to be identified was that for glucocorticoid receptors, which was called a glucocorticoid responsive element or GRE. The consensus GRE sequence is GGTACAnnnTGTYCY with the perfect half site being TGTTCT. Somewhat surprisingly, each of the classical steroid receptors except the estrogen receptor was found to act through the same HRE, being the sequence of the GRE [3–5]. Despite these differences in specific DNA binding sites, the binding to nonspecific DNA sequences by glucocorticoid, progesterone (PR) and estrogen receptors was similarly reduced by Sephadex G-50 chromatography. In each case, the original levels of DNA binding were restored by a low molecular weight factor(s) (3.0–0.7 kDa) that was present in cytosol and enriched in nuclear extracts, suggesting the presence of two subpopulations of receptors, one of which required factor(s) for effective DNA binding [6–8].

Numerous reports exist of factor-mediated increases in the DNA binding of steroid receptors. GREs and HREs usually do not exist as naked DNA in intact cells. Instead, they are commonly present in nucleosomes [9–12]. While the presence of chromosomal proteins has been reported to reduce the amount of receptor–steroid complex binding to DNA [13], the presence of nucleosomes usually decreased the affinity and increased the specificity of glucocorticoid receptor and PR binding [11, 14]. Other proteins and cellular factors have been reported to augment receptor binding to DNA and/or chromatin by a variety of mechanisms. Retinoid X receptors promote the HRE binding of those members of the steroid receptor superfamily that are in the nuclear receptor subfamily by forming heterodimers (Ref. [15]; reviewed in [16]). A 45 kDa single-stranded DNA-binding protein augmented the binding of the purified estrogen receptor to its responsive element [17]. Insulin degrading enzyme (IDE) is a 110 kDa protein that elevated the DNA binding of fragments of androgen and glucocorticoid receptors, but not the full length proteins expressed in baculovirus [18]. The DNA binding affinity of PRs was magnified about 10-fold by the 28 kDa protein HMG-1, possibly by helping to deform the DNA to a bent conformation that is beneficial for receptor binding [19]. Binding to nuclei and chromatin, but not DNA, was increased by a factor called ASTP, which may associate with the histones of nucleosomes to create a higher affinity site [20]. Finally, a protein could bind to DNA and create a new surface to which the receptor would associate with increased affinity, as has been proposed for the indirect binding of glucocorticoid receptors to AP-1 DNA sites via interaction with DNA-bound jun-fos dimers [21].

Previous studies with auxiliary DNA binding protein(s) have not addressed the question of whether the increased receptor binding to DNA resulted from effects on the entire population, or only a subpopulation, of a given receptor. In contrast, our ubiquitous low molecular weight factor(s) (0.7–3.0 kDa) was proposed to influence a subpopulation of receptors [6–8]. However, our studies were conducted with the nonspecific acceptor, calf thymus DNA. Nonspecific DNA binding is biologically relevant in that it acts as a buffer for specific binding, so that increases in the amount of activated receptor complexes result in a gradual increase in specific DNA binding and regulated gene expression [22]. Furthermore, nonspecific DNA binding permits the rapid formation and dissociation of receptor-DNA complexes that are needed for effective searches of the biologically active HREs amongst all of the inactive DNA sites [23, 24]. Nevertheless, it was not known whether the effects of the low molecular weight factor(s) on nonspecific DNA binding would be observed with specific DNA binding.

The purpose of the current investigation was to determine whether the low molecular weight factor(s) similarly affected the binding of receptors to specific DNA sequences, i.e. HREs. Such studies are prerequisites for an assessment of a possible role of receptor heterogeneity in receptor-mediated control of gene transcription. We also wanted to characterize the factor(s). We chose PRs for the present study because earlier results revealed a more robust response to the factor(s) with PR [8]. The DNA binding was assayed with a modified ABCD assay, which allowed a direct quantitation of receptor binding to DNA in solution, conditions that more closely approximate those found inside an intact cell. This system further permitted analysis of the effect of factor(s) on the kinetics of DNA binding and a test of the hypothesis that the receptor subpopulations for PR corresponded to the A and B isoforms.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0°C.

Chemicals

Nonradioactive dexamethasone (Sigma), nonradioactive R5020 (Du Pont-New England Nuclear), and [^{3}H]R5020 (85 Ci/mmol, Du Pont-New England Nuclear) were commercially available. Other purchased reagents were TAPS and HEPES (Ultrol grade, Calbiochem); SDS (Research Genetics); high and low molecular weight markers for SDS-polyacrylamide gels (Pharmacia); other reagents for SDS-polyacrylamide gel electrophoresis, including Coomassie Blue R-250 and EIA grade Tween 20 and 0.2 μ nitrocellulose membrane (Bio-Rad); fluorescent Ult-Emit

autoradiography marker and enhance or enlightning for fluorography (Du Pont-New England Nuclear); Protogel and Sequalgel 6 (National Diagnostics); enhanced chemiluminescence Western blotting detection kit (Amersham); ABC reagent for immunoperoxidase staining of Western blots (Vector Laboratories); TRIS and glycine (ICN Biochemicals); and, sodium molybdate (Baker Chemical). All other chemicals were obtained from Sigma. All ^{3}H-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic cpm-to-dpm conversion.

Oligonucleotides

The wild type biotinylated GRE/PRE oligonucleotide was 5′-XGATC**CTGTACAGGATGTTCTAGCTAC**A-3′ (X = biotin, bold sequence is that of the GREII of the rat TAT gene); the mutant PRE oligonucleotide was 5′-XGATC**CTGT*T*CAGGATG*A*TCTAGCTAC**A-3′, which contains two mutations that have been found to severely reduce PR binding to a PRE [25]. A long form, biotinylated oligonucleotide was 5′-X**GATCCGATCC**GATCCTGTACAGGATGTTCTAGCTACA**AGCTACAAGC**-3′ contained ten additional bases (bold) at either end of the wild type PRE oligonucleotide. Plus and minus strand oligonucleotides were prepared by GIBCO-BRL and stored in 10 mM Tris, 1 mM EDTA, 150 mM NaCl (pH 8.0 at R.T.). Double stranded DNA was prepared by heating (15 min at 65 or 90°C), followed by slow cooling. In most cases, the double stranded DNA was separated from single stranded DNA on 20% acrylamide gels. In some cases, however, no separation was made as the residual single stranded DNA did not alter the binding properties (data not shown).

In vitro translated receptors

Plasmids containing the A (hPR2) and B (hPR1) forms of human PR under the control of the SP6 promoter [26] were a gift from Dr Hinrich Gronemeyer (Strasbourg) and were transcribed/translated *in vitro* with Promega's TNT® coupled reticulocyte lysate system according to the manufacturer's specifications.

Cells

Rat hepatoma tissue culture cells (HTC) were grown in Swim's S77 medium supplemented with 5% newborn calf serum, 5% fetal calf serum and 0.03% glutamine [27]. MCF-7 human breast cancer cells were cultured at 37°C in Richter's improved minimal essential medium (zinc option with folic acid) and collected as previously described [8]. T47D human breast cancer cells were grown at 37°C in either T-flasks or 2 l roller bottles in Dulbeco's modified eagle's medium with high glucose (4.5 g/l, 2 mM glutamine and 60 ng/ml insulin) and 10% heat inactivated fetal calf serum (Biofluids, Rockville, MD). T-flasks were kept in a 5% CO_2 atmosphere. The roller bottles containing 100 ml of medium were firmly capped and rotated at 0.05 rpm, with complete medium changes every 2–3 days. Cells were harvested by washing with 20 ml of phosphate buffered saline (PBS) at R.T., incubating with 5 ml of 0.05% trypsin/0.02% EDTA for 10–15 min at 37°C and collected in 25 ml of cold medium. The contents of four roller bottles were combined in one 250 ml centrifuge tube. After a low speed centrifugation (4000*g*/5 min/0°C), the pellets were washed twice with 100 ml of 0°C PBS and stored at −80°C until needed.

Preparation and activation of receptors

Cytosolic receptors were prepared by freeze–thaw lysis of cell pellets [28] in an equal volume of Hepes buffer (20 mM Hepes, 1 mM EDTA, 10% glycerol, pH 7.5 at 0°C). The pellet from a 32,000*g* centrifugation (15 min/0°C; five roller bottles of cells per tube) was used to prepare nuclear factor(s) (see below) while the supernatant was recentrifuged at 200,000*g*/90 min/0°C and then stored at ≤ -80°C until used. These cytosolic receptor solutions were diluted to 60 vol% in pH 7.5 Hepes buffer, bound with 90 nM [^{3}H]R5020 plus 20 μM nonlabeled dexamethasone [to block binding of R5020 by glucocorticoid receptors but often omitted with T47D cytosols since Western blotting showed that T47D cytosol did not contain glucocorticoid receptors (data not shown)] $\pm$20 μM nonlabeled R5020 for 2 h. The receptors were then activated by first diluting with an equal volume of pH 7.5 Hepes buffer and then heating at 20–25°C for 10 min. An aliquot (0.5 ml) was chromatographed over a 9.1 ml Sephadex G-50 column equilibrated with pH 7.5 Hepes buffer and the peak 0.5 ml fraction of protein was used as Sephadex G-50′d cytosol.

Preparation of nuclear factor(s)

Crude nuclear factor(s) was prepared as before [6] by incubating each pellet, from either five roller bottles of T47D cells or 10^{9} HTC cells, with 6 ml of 0.5 M NaCl in pH 7.5 Hepes buffer for 1 h after an initial vigorous vortexing followed by intermittent vortexing. After centrifugation (12,000*g*/10 min), the factor(s) was precipitated with 0.65 volumes of saturated $(NH_4)_2SO_4$ (final concentration is 40%) followed by centrifugation (3000*g*/10 min). Each pellet was resuspended in 4 ml of pH 7.5 Hepes buffer, frozen in dry ice, and stored at −80°C. Partially purified factor(s) was made fresh by desalting on a Sephadex G-10 column that had been equilibrated in pH 7.5 Hepes buffer, heating for 10 min at 100°C and passing the supernatant (2000*g*/3 min) through an Amicon C3 membrane by centrifugation at about 3000*g*/30 min.

DNA binding assays

Minicolumn assays, consisting of passing the various receptor solutions through a calf thymus DNA-cellulose column and then a DEAE-cellulose column, were performed as described [6]. The binding of non-receptor species to the DNA-cellulose and DEAE-cellulose minicolumns, as determined by species labeled by [^{3}H]R5020 plus 2×10^{-5} M of nonlabeled dexamethasone and of nonlabeled R5020 was generally less than 5% and therefore ignored.

Conventional ABCD assays were conducted by incubating 100 μl receptor preparations with 1–20 pmol of double stranded PRE oligonucleotide for 1 h followed by a 1 h incubation with 30 μl of 50% slurry of streptavidin-agarose in pH 7.5 Hepes buffer on a Belco rotating drum at 11 rpm. The steroid–receptor–biotinylated DNA–streptavidin–agarose complexes were pelleted (15,000*g*/30 s), washed with two 0.5 ml aliquots of pH 7.5 Hepes buffer and then placed in a scintillation vial for counting.

Modified ABCD assays involved prebinding 500 pmol of double-stranded biotinylated PRE, or mutant PRE, oligonucleotide to 1.0 ml of a 50% slurry of streptavidin–agarose beads for 2 h on the Belco rotating drum. The beads were then washed with 10 ml of 10 mM NaCl in pH 7.5 Hepes buffer, then twice with 10 ml of pH 7.5 Hepes buffer without salt and resuspended in an equal volume of pH 7.5 Hepes buffer. Aliquots (20 μl) of the 50% solution of PRE-streptavidin–agarose (containing 10 pmol PRE) were incubated with 40 μl of receptor $\pm$ 40 μl of nuclear factor(s), or different salts, in pH 7.5 Hepes buffer, (total volume is 100 μl) for the desired length of time and then processed for counting as above in the conventional ABCD assay. In both ABCD assays, the nonspecific binding to streptavidin–agarose was $\leq$3% and was ignored.

SDS-polyacrylamide gels and Western blotting

Samples diluted 1:2 in 2 $\times$ SDS buffer were analyzed on constant percentage acrylamide gels (between 7 and 14%, depending on the molecular weight range being examined, with a 1:37.5 ratio of bisacrylamide to acrylamide) run in a water-cooled (15°C) Protean II slab gel apparatus (Bio-Rad) at 35 mA/gel. Gels were fixed, stained, marked at the positions of the molecular weight markers with fluorescent paint, and fluorographed for 1–2 weeks at −80°C as described [29]. Electrophoretic transfer from SDS-polyacrylamide gels to nitrocellulose, conducted at 400 mA for 5 h, or 120 mA overnight, in a Trans-Blot (Bio-Rad) apparatus, followed by staining with Ponceau S to monitor the protein transfer, incubation with primary and secondary antibodies, and visualization by enhanced chemiluminescence, were conducted as described [29], usually with the following modifications. After incubating the filters with blocking buffer [0.05% Triton, 0.25% gelatin, 3% bovine serum albumin in 1× HEN buffer (10 mM HEPES, 1 mM EDTA, 30 mM NaCl)] for 2 h with shaking, the filters were washed in 4 M urea wash (4 M urea, 50 mM NaCl, 5 mM EDTA) for 1 h with shaking, followed by three 5 min washes with 25 ml of wash buffer (0.05% Triton, 0.25% Gelatin in 1× HEN buffer). The filter was incubated overnight at 4°C with shaking with antiprogesterone antibody AB52 (5 μg/ml in 1× HEN buffer plus 0.05% Triton, 0.25% Gelatin, 1% BSA) [30].

Analysis of scanned gel images

Western blots were digitized with a Microtek Scanmaker III with transparent media adapter and analyzed on a Macintosh Quadra 800 computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868). When unequal areas of signal were to be analyzed, NIH Image was used to select a region around the band of interest and to quantitate the *area* and *mean signal per unit area* for that region. The automatic background subtract feature of NIH Image could not be used since it computed the background as being the average signal of the selected region. Therefore, the background had to be calculated manually by selecting other regions, not necessarily of equal area, in the vicinity of the desired band and determining the average of the analyzed *mean signal per unit area*. The total intensity for the band of interest was then equal to [(*mean signal per unit area* of the selected band) — (*mean signal per unit area* of background)] $\times$ (*area* of selected band).

RESULTS

PR from T47D and MCF-7 cells both display subpopulations for nonspecific DNA binding

Our earlier studies were conducted with PR from human MCF-7 cells [8]. In order to obtain higher concentrations of PR, we chose human T47D cells, which have very high levels ($\geq$100 nM) of endogenous PR [25]. As expected, the DNA binding properties were independent of the source of the receptors. The ability of the low molecular weight factor(s) from rat HTC cells to increase the nonspecific DNA binding of T47D cell PR was almost identical to that of MCF-7 cell PR [Fig. 1(a)]. Nuclear factor from T47D and HTC cells was indistinguishable (data not shown) and therefore was used interchangeably. The factor-increased DNA binding was observed both for the receptor–steroid complex solutions of activated cytosol, which contained some endogenous factor(s), and for the cytosolic complexes that had been passed

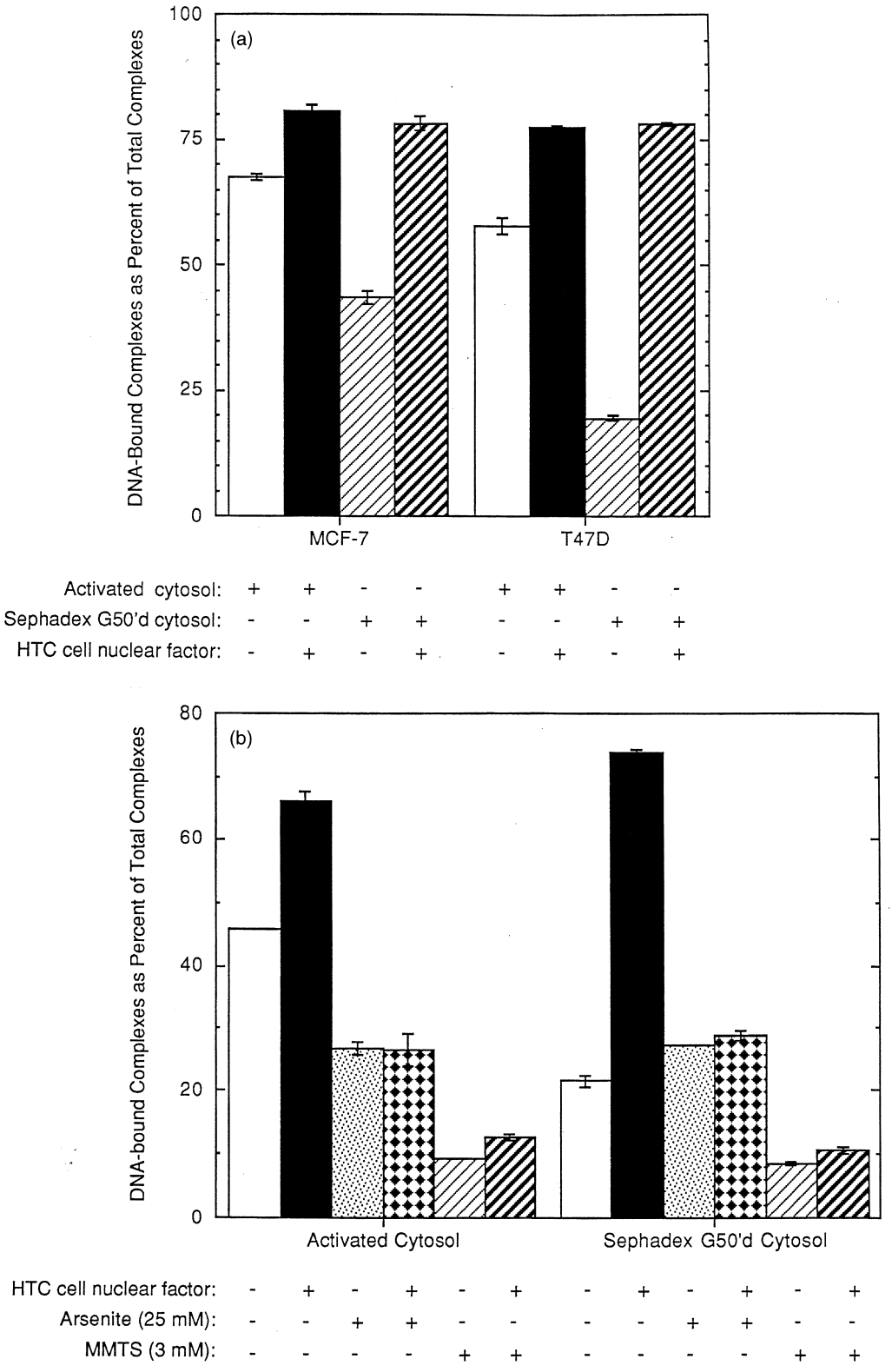

Fig. 1. Properties of T47D cell receptor binding to DNA–cellulose minicolumns. (a) Binding of PR complexes from MCF-7 and T47D cells and (b) effect of arsenite and MMTS on DNA binding of T47D cell receptors before and after Sephadex G-50 chromatography. Activated and Sephadex G-50 chromatographed samples of PR bound by [^{3}H]R5020, with or without the indicated treatments of nuclear factor(s), arsenite, or MMTS, were loaded onto duplicate minicolumns and the binding to DNA and DEAE–cellulose columns was determined. The average binding (±range) to DNA, as a percentage of total receptors recovered on DNA and DEAE–cellulose columns, was then plotted as described in Section 2. Similar results were obtained in at least one other experiment.

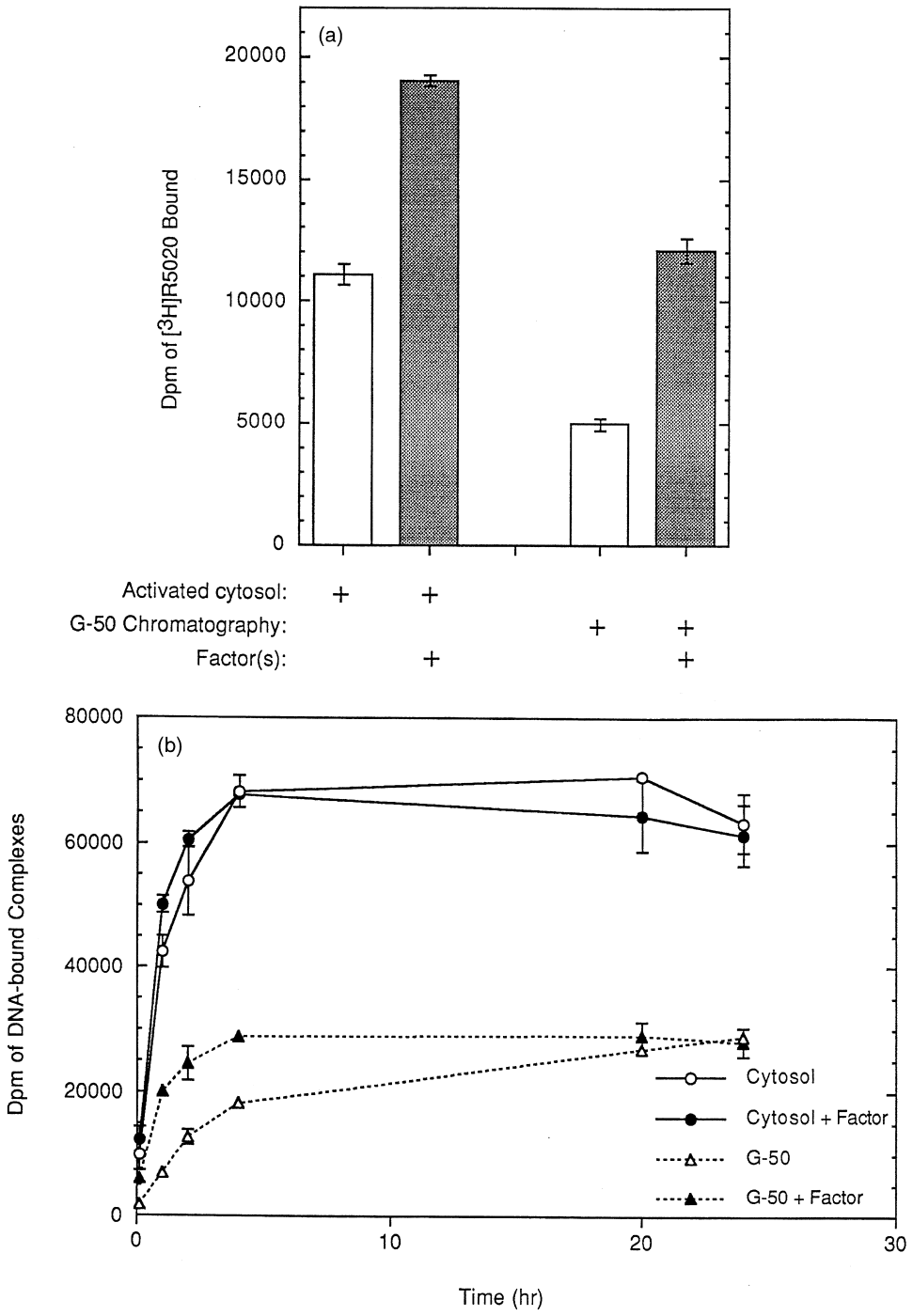

Fig. 2. PR binding to PRE in the modified ABCD assay. (a) Effect of nuclear factor(s) on PR binding to PRE. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were treated, before or after Sephadex G-50 chromatography, with pH 7.5 Hepes buffer ± nuclear factor(s) and incubated with immobilized PRE for about 45 min, as described in Section 2.The data are plotted as the average binding (±range). Similar results were obtained in at least four other experiments. (b) Effect of nuclear factor(s) on the kinetics of PR binding to PRE. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were treated, before or after Sephadex G-50 chromatography, with pH 7.5 Hepes buffer ± nuclear factor(s) and incubated with immobilized PRE for the indicated times, as described in Section 2. The data are plotted as the average binding (±range). Similar results were obtained in at least one other experiment. (c) Effect of competing DNA on the kinetics of dissociation of PRE-bound PR. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were incubated with immobilized PRE for 4 h. Aliquots of nonbiotinylated PRE were added and the residual binding determined at the indicated times. Similar results were obtained in two other experiments.

through a G-50 Sephadex column to remove the factor(s). Sodium arsenite blocked the DNA binding of those receptors that required factor(s) for binding but had no effect on the binding capacity of receptor–steroid complexes already depleted of factor(s) by Sephadex G-50 column chromatography [Fig. 1(b)]. Therefore, two different methods can be used to prevent the DNA binding of a PR subpopulation that requires factor(s) for binding: Sephadex G-50 chromatography or 25 mM sodium arsenite. Finally, in contrast to the behavior of glucocorticoid receptors but similar to PR from MCF-7 cells [7, 8], methyl methanethiolsulfonate (MMTS) prevented essentially all of the DNA binding of T47D cell PR with or without added factor(s) [Fig. 1(b)]. The small amount of MMTS-resistant binding appears to be due to the nonspecific binding of PR to the cellulose matrix of the columns [8].

Effect of factor(s) on PR binding to PRE in the modified ABCD assay

The conventional ABCD assay for steroid receptors binding to DNA was essentially that described by Murdoch *et al.* [31]. A 27 bp double stranded oligonucleotide with one biotinylated nucleotide at each 5′-end was used. This oligonucleotide contained 22 bp of the second GRE of the TAT gene [32] and is biologically active with PR [33].

About 45% of the available PR bound to ~100 nM PRE (10 pmol/100 μl) DNA after 60 min. However, the effect of factor(s) was to decrease the total binding (data not shown). This appeared to be due to an instability of the bound complexes at short times of incubation with low concentrations of PRE, which reduced the total binding to values that were stable at times greater than 1 h. This instability was not further investigated, especially since the mandatory 1 h post-incubation with streptavidin–agarose was an undesirable property of the conventional ABCD assay that made kinetic experiments difficult to interpret. For this reason, we developed a modified ABCD assay in which the biotinylated DNA was prebound to streptavidin before the addition of receptor–steroid complexes, thereby permitting workup after any length of time for PR binding.

As with the conventional ABCD assay, 10 pmol of PRE (0.1 μM) was sufficient for maximal levels of binding (data not shown). A 3–4 h incubation of activated cytosol plus factor(s) gave 88 ± 16% (±S.D., $n = 4$) of the PR binding seen on minicolumns. Thus, the modified ABCD assay detected a large amount of the PR that can bind to DNA. A mutant oligonucleotide containing two mutations that were detrimental for PR binding [25] displayed specific binding that was less than 50% of that seen with the wild type PRE (data not shown; see also below).

Added low molecular weight HTC or T47D nuclear factor(s) increased the total specific binding to PRE of activated PR complexes. This was observed for both the activated complex solutions, which contained some factor(s), and for Sephadex G-50 chromatographed complexes, which had been depleted of factor(s) [Fig. 2(a)]. Thus, the PR binding detected in the modified ABCD assay, which is to high affinity and transcriptionally active sites, is affected by added factor(s). It should be realized that the method of data presentation in Fig. 2 is different from that in Fig. 1 because there is no way to quanti-

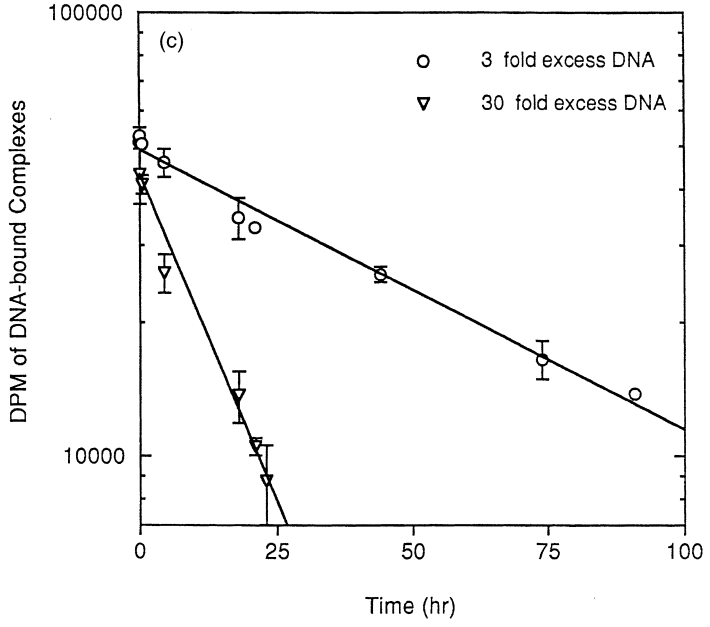

Fig. 2(c). ***(cont.).***

Table 1. *Effect of factor(s) on kinetics of PR binding to PRE in modified ABCD assay*

Sample	Half-time of association (h)	
	without factor(s)	with factor(s)
Activated PR	0.61 ± 0.16 (4)	0.50 ± 0.16 (4)
	[$P < 0.03$]	
Sephadex G-50 chromatographed PR	2.06 ± 0.33 (3)	0.64 ± 0.17 (4)
	[$P < 0.0007$]	

Experiments such as those of Fig. 2(b) were analyzed to determine the half-time of PR association with PRE DNA. The data listed are ±SD for the number of experiments in parentheses. The *P* values (unpaired student *t*-test for Sephadex G-50 and paired Student-*t* test, to compensate for parallel changes in the individual rates between experiments for Activated PR) are listed below the relevant sets of data.

tate the receptor complexes that do not bind to DNA in the ABCD assay, as was achieved in Fig. 1 from the binding to DEAE–cellulose.

The data of Fig. 2(b) show that the major effect of factor(s) was to increase the rate of PR complex binding. As listed in Table 1, factor(s) was able to cause statistically significant increases in the kinetics of binding of activated PR complexes both before and after Sephadex G-50 chromatography. Equally informative is that the half time of PR binding of Sephadex G-50′d complexes was decreased to almost the same value as observed for activated PR with added factor(s). Thus, we propose that the PR binding to PRE oligonucleotides in the absence of factor(s) (i.e. after Sephadex G-50 chromatography) reflects the cumulative binding of two equally abundant subpopulations. One, which bound completely and rapidly during the first approximately 4 h in the absence of factor(s), and a second subpopulation that required 20–24 h for maximal binding. No plateau was seen at intermediate times of binding, though, and curve fitting analyses of data, such as in Fig. 2(b), was unable to determine whether one or two binding components existed (see Section 4).

The PRE-bound PR was very stable in the modified ABCD assay. The half time for dissociation was $\geq$10 h in the presence of a 3-fold excess of nonbiotinylated DNA [Fig. 2(c)]. It should also be noted that the half time of receptor–steroid complex dissociation in Fig. 2(c) was very dependent on the amount of nonbiotinylated DNA present and was significantly decreased by higher concentrations of competing DNA (see Section 4).

Effects of arsenite, or MMTS, on PR binding to PRE ± factor(s)

We had previously reported that sodium arsenite and MMTS were capable of selectively inhibiting the binding of apparent subpopulations of glucocorticoid receptors [7] and PR [8] to nonspecific DNA on minicolumns. We therefore examined the effects of arsenite and MMTS in the modified ABCD assay at short incubation times where the only binding observed would be of those PR complexes thought not to require factor(s). As shown in Fig. 3(a), arsenite concentrations between 0.5 and 3 mM blocked the binding of about half of the PR complexes of activated cytosol in a manner that could not be overcome by added factor(s). Higher arsenite concentrations eliminated almost all DNA binding. Significantly, no concentration of arsenite up to 25 mM had any appreciable effect on the binding of PR that had already been chromatographed over Sephadex G-50 to remove factor(s) [Fig. 3(a)]. Furthermore, 25 mM arsenite blocked the ability of added factor(s) to increase the binding of Sephadex G-50 chromatographed PR. Low concentrations of MMTS eliminated virtually all DNA binding [Fig. 3(b)]. These results are very similar to those observed for PR binding to nonspecific DNA on minicolumns [8]. These data also support the existence of two PR subpopulations, one of which requires factor(s) for DNA binding and does not bind to DNA in the presence of arsenite.

Identification of factor(s) as $(NH_4)_2SO_4$ and its effect on the kinetics of PR binding

Analyses of Sephadex G-10 chromatographs of solutions of protein, NaCl and $(NH_4)_2SO_4$ demonstrated that $(NH_4)_2SO_4$ by itself behaves atypically and is partially excluded from the matrix that should include all species of M_r<700 Da [Fig. 4(a)]. The protein of the nuclear extract appeared in the void volume and the NaCl in the included volume, as expected. However, $(NH_4)_2SO_4$, either by itself or in the presence of the proteins in the resuspended nuclear extract after $(NH_4)_2SO_4$ precipitation, eluted at an intermediate position that overlapped with the collected fractions of Sephadex G-10 chromatographed nuclear extract [see solid bar in Fig. 4(a)]. Thus, the 'nuclear factor(s)' fractions would contain low concentrations of $(NH_4)_2SO_4$. A close examination revealed that the ability of individual Sephadex G-10 column fractions of nuclear extract to increase the specific DNA binding of Sephadex G-50 chromatographed PR in the modified ABCD assay exactly correlated with the increased ionic strength of the fractions that was caused by the aberrant chromatographic behavior of $(NH_4)_2SO_4$ (data not shown). Concentrations of $(NH_4)_2SO_4$ as low as 5 mM were sufficient to cause increased DNA binding by Sephadex G-50 chromatographed PR and 15 mM was usually sufficient for maximal effects. Low concentrations of some other salts (e.g. 15–25 mM NaCl and KCl) were also able to cause increased DNA binding [Fig. 4(b)]. However, this effect was not simply one of increased ionic strength as 0.5–25 mM sodium arsenite had no effect on Sephadex G-50 chromatographed PR [see Fig. 3(a)].

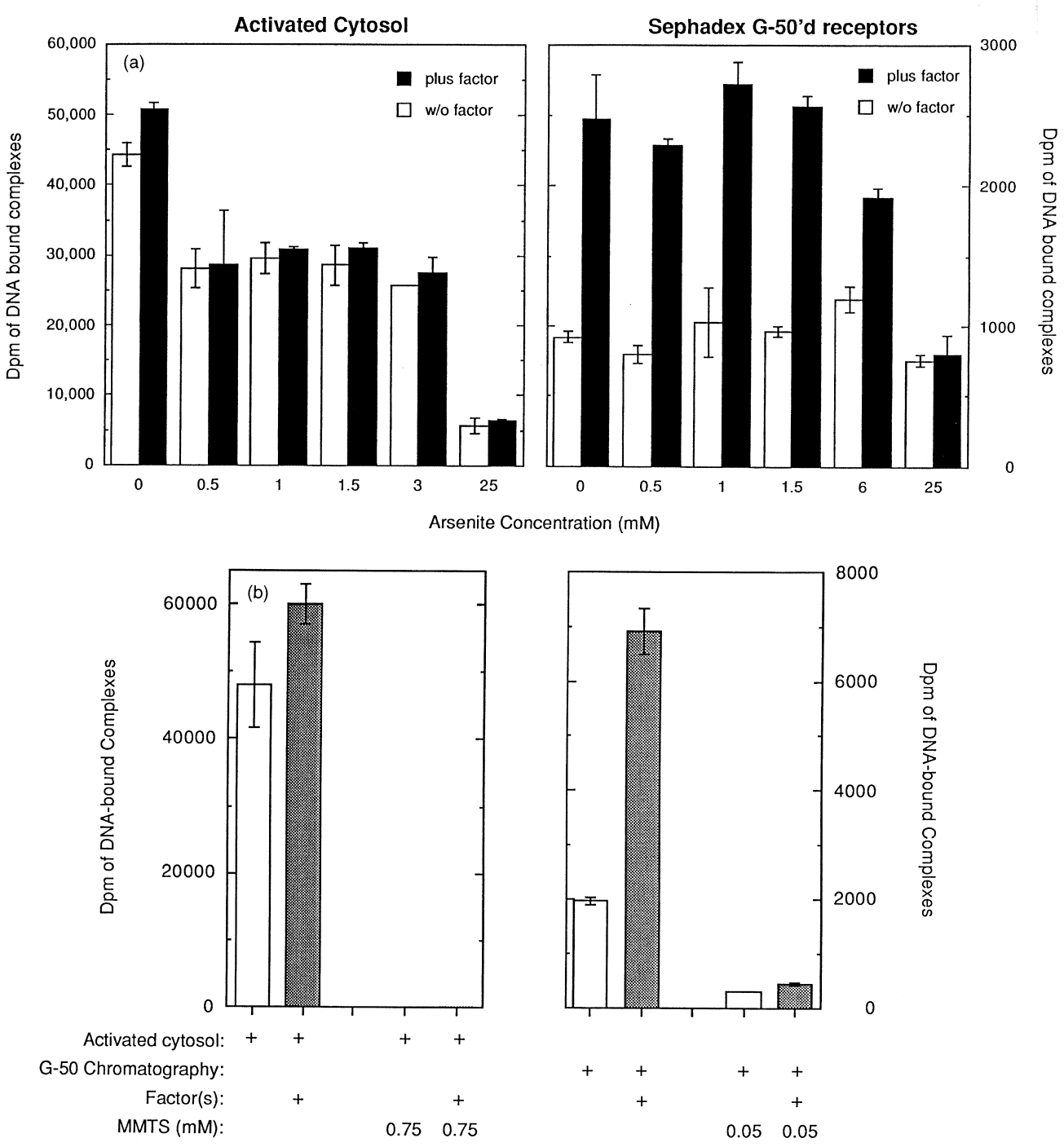

Fig. 3. Effect of arsenite (a) and MMTS (b) on PR complex binding to PRE in the modified ABCD assay. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were treated, before or after Sephadex G-50 chromatography, with pH 7.5 Hepes buffer ± nuclear factor(s) and the indicated concentrations of either arsenite or MMTS for 15 min, as described in Section 2. The data are plotted as the average binding to immobilized PRE (±range). Similar results were obtained in at least one other experiment.

To further establish that the nuclear factor(s) was, in fact, $(NH_4)_2SO_4$ that was migrating anomalously on Sephadex G-50 columns, we examined the kinetics of PR binding to PRE in the presence of low concentrations of $(NH_4)_2SO_4$. Very similar results were seen when 15 mM $(NH_4)_2SO_4$ [Fig. 5(a)] or 50 mM KCl (data not shown) was employed instead of 'nuclear extract factor(s)' (cf. Figure 2(b)]. Thus, we conclude that the 'nuclear extract factor(s)' is $(NH_4)_2SO_4$ and that the 'factor(s)' removed from activated cytosol by Sephadex G-50 is salt. It should be noted that the ionic strength of activated cytosol

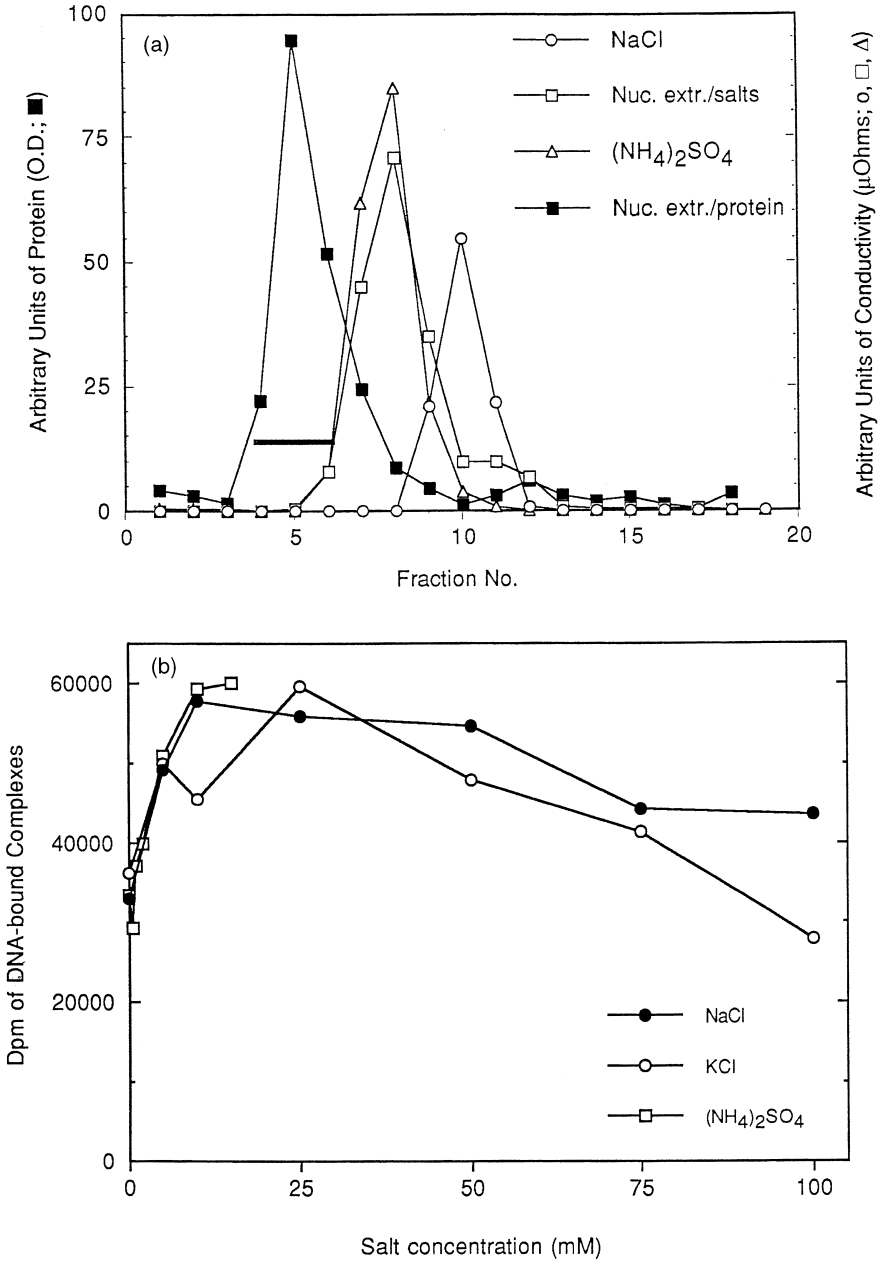

Fig. 4. Role of $(NH_4)_2SO_4$ and other salts in the increased PR binding to PRE. (a) Sephadex G-10 chromatographic properties of nuclear extract, $(NH_4)_2SO_4$, and KCl. The indicated samples (0.5 ml) were loaded onto Sephadex G-10 columns (9 ml volume in 1 cm diameter columns) and 0.5 ml fractions were collected and analyzed for protein content (■) or conductivity (□, nuclear extract; △, $(NH_4)_2SO_4$; ○, NaCl). The collected nuclear factor(s) usually consisted of the 1.9–3.1 ml fractions of eluant (see bar at the base of the elution profiles). (b) Ability of various salts to cause increased PR binding to PRE. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were treated, after Sephadex G-50 chromatography, with the indicated concentrations of salt for 45 min, as described in Section 2. The data are plotted as the average binding to immobilized PRE. Similar results were obtained in at least one other experiment for $(NH_4)_2SO_4$ and KCl.

solutions was found to be equal to that of about 35 mM $(NH_4)_2SO_4$.

Interestingly, the effect of increasing salt was very different for PR binding to wild type and mutant PREs. Low concentrations of KCl increased the binding of Sephadex G-50 chromatographed PRs to both acceptors, but the nonspecific binding to the mutant PRE was much more sensitive to increased salt [Fig. 5(b)]. As a measure of the specificity of binding at different salt concentrations, the ratio of PR binding to specific vs mutant PRE was plotted [dashed line in Fig. 5(b)]. Thus, while the total binding of PR to a biologically active PRE was highest at 5–20 mM KCl, the greatest specificity of binding was seen at about 75 mM KCl.

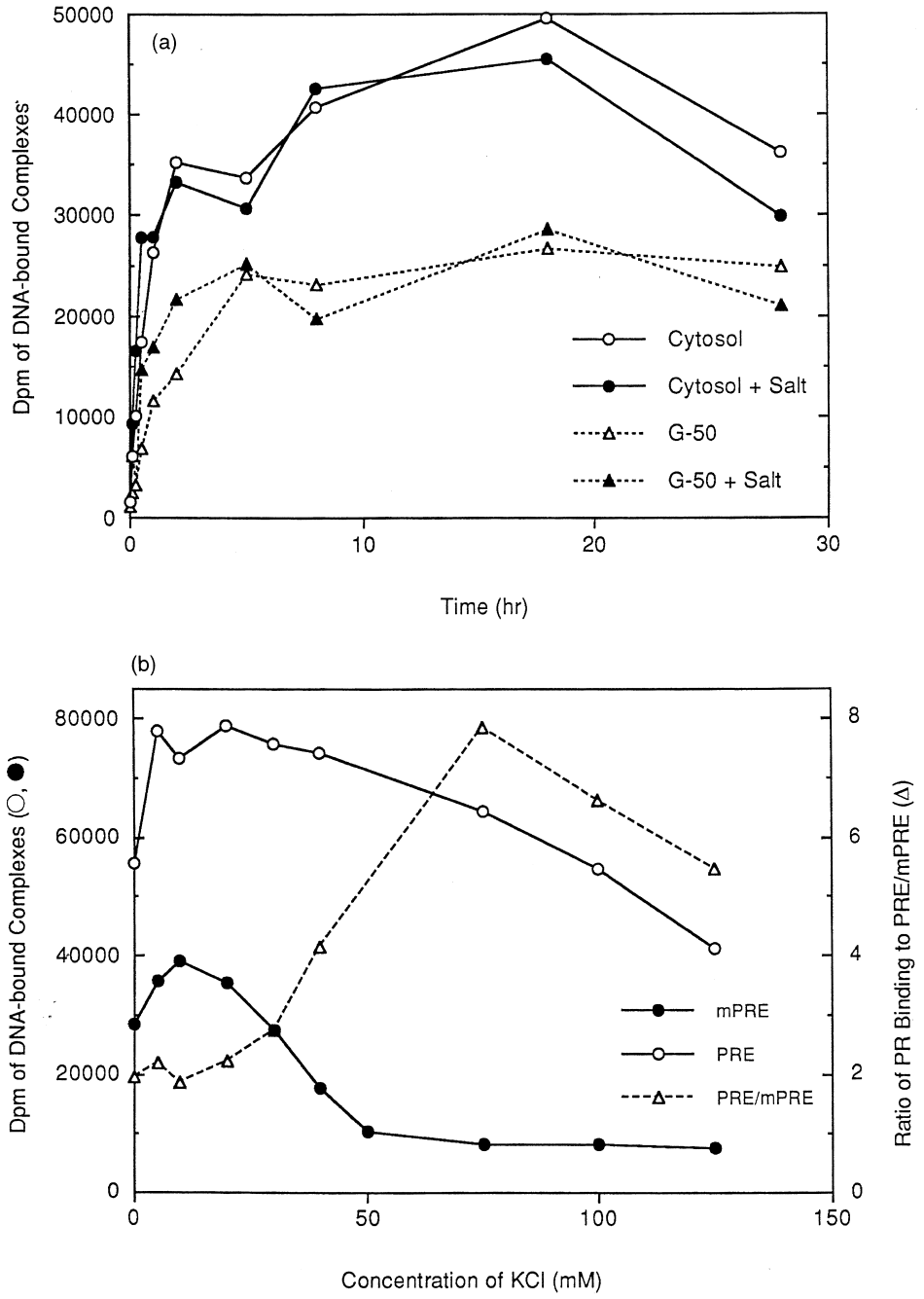

Fig. 5. Effect of salts on the kinetics and specificity of PR binding to PRE. (a) Effect of 15 mM $(NH_4)_2SO_4$ on the kinetics of association of PRs. Duplicate samples of activated, [^{3}H]R5020-bound T47D cell PR were treated, before or after Sephadex G-50 chromatography, with pH 7.5 Hepes buffer ± nuclear factor(s) and incubated with immobilized PRE for the indicated times, as described in Section 2. The average range was ≤6%, but was omitted for clarity. Similar results were obtained in at least one other experiment. (b) Effect of KCl concentration on the binding of PR to wild type and mutant PREs in the modified ABCD assay. Single samples of Sephadex G-50 chromatographed, activated, [^{3}H]R5020-bound T47D cell PR in pH 7.5 Hepes buffer were incubated with immobilized wild type, or mutant, PRE in the presence of the indicated concentrations of KCl for 45 min, as described in Section 2. The dpm of bound radioactivity (left hand axis) and the ratio of specific to nonspecific binding (PRE/mPRE) (right hand axis) were then plotted for each salt concentration. Similar results were obtained in one other experiment.

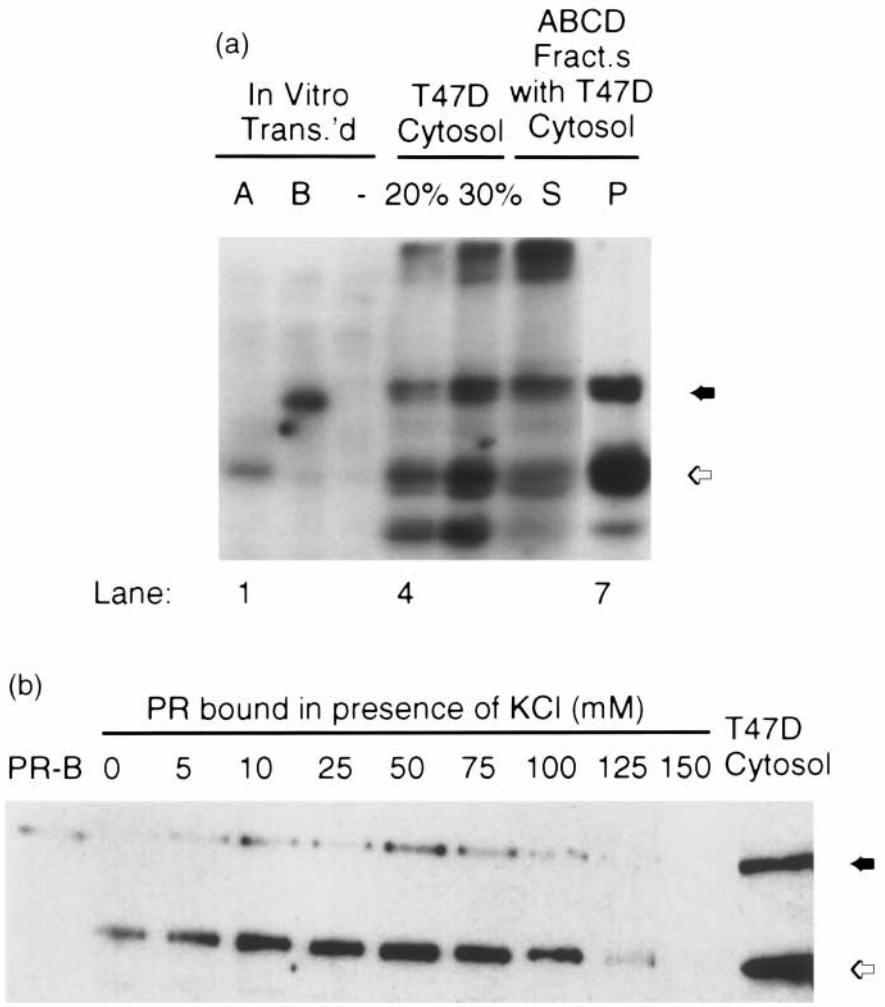

Fig. 6. Western blot analysis PR isoform binding to PRE in the modified ABCD assay. (a) A large scale (5×) modified ABCD assay was performed with activated T47D PR complexes (lanes 4 and 5 = 20 and 30% of cytosol by volume) and *in vitro* translated PR-A (lane 1) and PR-B (lane 2; lane 3 contains an equal volume of unprogramed reticulocyte lysate). After a 1 h incubation at 0°C, the agarose beads were pelleted by centrifugation and an aliquot of the supernatant of tubes containing T47D cytosol (S; lane 6) was removed for gel analysis. The pellet was washed with 2 × 2.5 ml of pH HEPES buffer and extracted with 100 μl of extraction buffer (20 mM CHAPS, 0.5 M KCl and 20 mM DTT in pH 7.5 HEPES buffer) for 1 h/0°C. This procedure removed 50–60% of the specifically bound dpm, as determined by counting of the extracted pellets. Pellet extract was diluted with 2× SDS sample buffer, separated on a 7% SDS–polyacrylamide gel, and Western blotted with AB52 anti-PR antibody, which recognizes both PR isoforms, as described in Section 2. Similar results were obtained in at least nine additional experiments. (b) PR isoform binding to PRE after Sephadex G-50 chromatography in the presence of various concentrations of KCl. Samples of Sephadex G-50 chromatographed, activated, [^{3}H]R5020-bound T47D cell PR were incubated in the presence of the indicated concentrations of KCl and processed as above in (a). The lane labeled 'T47D Cytosol' contains the starting Sephadex G-50 chromatographed PR. In both (a) and (b), the position of PR-A and -B are indicated by the open and filled arrows, respectively. Similar results were obtained in a second experiment.

PR isoform binding specificity in the modified ABCD assay

Human and chick PR exist as two isoforms that are thought to arise from alternative translational start sites from the same [34] or different [26, 35] mRNA transcripts. While no differences in the DNA binding of these isoforms has been noted in the common gel shift assays [19, 25], the shorter PR-A was originally thought to have a much higher affinity for DNA in conventional solution binding assays [36]. In order to determine whether there might be selective binding of the A or B isoforms of PR in the modified ABCD assay, we extracted the pellets of binding assays, separated the receptor isoforms on SDS–polyacrylamide gels, visualized the receptors by Western blotting coupled with enhanced chemiluminescence, and quantitated each isoform by computer analysis with the program NIH Image. As shown in Fig. 6(a), both A and B isoforms of activated cytosol bound to the PRE in this assay. However, there was a statistically significant preference for the DNA binding of PR-A

in activated cytosols. Thus, PR-A comprised 70 ± 6% of the total receptors (PR-A:PR-B = 2.3:1) in activated cytosol but constituted 79 ± 12% of the total receptors (PR-A:PR-B = 3.8:1) that were extracted from the biotinylated PRE/streptavidin–agarose pellets (±SD, $n = 9$; $P = 0.02$ in Mann–Whitney two-tailed test). This preferential binding of PR-A was maintained with a long form of the biotinylated PRE, which contained an additional 10 bases between the usual PRE oligonucleotide and the biotin (data not shown). This result eliminated the possibility that the preferential binding of PR-A might have been due to some steric interference for the binding of the larger PR-B due to the short distance between the PR binding site in the PRE oligonucleotide and the biotin–streptavidin–agarose complex. In Sephadex G-50′d cytosols, there was again a marked preference for the binding of PR-A over PR-B in a manner that was relatively insensitive to changes in KCl concentration. Thus, PR-A constituted 69% of the complexes in Sephadex G-50′d cytosol (PR-A:PR-B = 2.2:1), but over 83% of the PRE-bound complexes were PR-A (PR-A:PR-B>4.9:1) over the range of 0–100 mM KCl [Fig. 6(b)]. These results were very similar to the preferential binding of PR-A seen for activated cytosol. These results also rule out the increased PRE binding of PR in the presence of additional salt as being due to a selective action of salt on either PR-A or PR-B. Whether or not there is a subpopulation of PR-A, PR-B, or both, that is affected by the higher salt concentrations cannot be determined from these data.

DISCUSSION

We have developed a modified avidin/biotin-coupled DNA (ABCD) binding assay to monitor the effects on the specific DNA binding of PR by our previously reported low molecular weight factor(s) from nuclear extracts. With this modified assay, we have demonstrated that the ability of factor(s) to increase the binding of Sephadex G-50 chromatographed PR to nonspecific DNA is general and was retained for PR binding to a biologically active PRE sequences. Surprisingly, the active component of nuclear extracts was found to be $(NH_4)_2SO_4$ that was not fully separated from the protein by Sephadex G-10 chromatography. The effect of this $(NH_4)_2SO_4$ was to accelerate the rate of DNA binding of an apparent subpopulation of PR. The PR-A isoform bound to a PRE in preference to PR-B at all salt concentrations examined. Thus, the ability of salt to increase the DNA binding kinetics of activated PR complexes did not correspond to a selective effect on either the PR-A or -B isoform but rather may reflect the action of salt on a subpopulation of one or both isoforms.

The presence of $(NH_4)_2SO_4$ in the void volume after Sephadex G-10 chromatography of $(NH_4)_2SO_4$ precipitated nuclear extracts was totally unexpected. The low molecular weight of $(NH_4)_2SO_4$ should have caused it to migrate in the included column volume along with the other salts [Fig. 4(a)]. We have not been able to find any mention of this behavior of $(NH_4)_2SO_4$ on Sephadex columns, but, it clearly cannot be ignored in DNA binding assays of PR. Given the similar ability of nuclear extracts to increase the binding of glucocorticoid [6, 7] and estrogen [8] receptors to nonspecific DNA sequences, we believe that the unexpected $(NH_4)_2SO_4$ was also responsible for the increased DNA binding of these receptors, presumably by again increasing the rate of binding. Conversely, the decreased magnitude and rate of DNA binding seen after Sephadex G-50 chromatography of cytosolic receptors would be due to the removal of endogenous salts [Fig. 4(b)]. The increased nuclear binding of Sephadex G-50 chromatographed complexes [6] presumably resulted from the higher ionic strength of the intranuclear compartment.

Many reports have appeared in the literature regarding the effect of salt on steroid receptor binding to DNA. Salt (KCl or NaCl) concentrations up to 100 mM increased the specific DNA binding of estrogen receptors [37], while higher concentrations decreased the nonspecific binding of estrogen and progesterone receptors [38]. Moderate salt concentrations (<100 mM) also decreased the binding of glucocorticoid receptors to nonspecific [27] and specific [39] DNA but increased the specificity of binding of GR to specific DNA sequences [40]. In this respect, our increased total DNA binding in the presence of some salts is not unique. However, we are not aware of previous demonstrations of differential effects of salt on the specific vs nonspecific binding of PR [Fig. 5(b)], indicative of increased selectivity of PR binding under physiological conditions. Furthermore, to the best of our knowledge, the ability of some salts to increase the rate of receptor binding to DNA is novel. This rate acceleration is not simply a general salt effect of increased ionic strength. The maximal effect of most active salts was seen at about 25 mM [Fig. 4(b)], while similar concentrations of sodium arsenite caused no significant increase [Fig. 3(a)] [8].

The precise mechanism of accelerated DNA binding by PR in the presence of many salts is not known, but is almost certainly different from the increased amount of DNA binding that can be effected by other species [41], such as the DNA-bending protein HMG-1 [19], a less well-defined factor of 50–75 kDa [42], and a Triton X100 extractable chromosomal protein, RBF-1 [43]. The increased binding kinetics with some salts might result from a selective effect on the PR-A or PR-B isoform. A salt-specific

effect on the DNA binding of PR-A would be especially interesting, as PR-A has been observed to act as a dominant negative inhibitor of both PR-B and other steroid receptors in some [44], but not all [45] situations. However, the data of Fig. 6(b) clearly show that the binding to PRE of neither PR-A nor PR-B was selectively affected by salt.

The increased rate of DNA binding in the presence of salt could be a property of all of the PR complexes present. In this case, $(NH_4)_2SO_4$ would increase the rate of binding of all complexes in a manner that is specifically blocked by sodium arsenite. However, two observations argue against this and for salt effecting the DNA binding of only a subpopulation of PR: (1) the plateau in the dose-response curve for arsenite inhibition of the DNA binding by activated PR [Fig. 3(a)] indicates that some PR complexes are relatively resistant to inhibition by arsenite and (2) the absence of inhibition by arsenite with complexes that were previously chromatographed over Sephadex G-50 [Fig. 1(b) and Fig. 4(a)] shows that the same population of PR can be prepared by two independent methods. These properties are identical to those seen in the binding of PR to nonspecific calf thymus DNA [8]. Thus, we conclude that the effect of salt on the kinetics of PR binding to nonspecific and specific DNA reflects an increase in the rate of binding for a subpopulation of PR as opposed to the recruitment of a population of PR that could not bind to DNA in the absence of salt. It should be noted that the ability of some salts, but not arsenite [Fig. 3(a)], to increase receptor binding to DNA is yet another example of a reagent, or process, that appears to have comparable effects on protein interactions with both nonspecific and specific DNA [24, 40, 46–50]. In fact, it has been suggested that nonspecific DNA binding is a good model for all features of specific DNA binding other than specific base contacts [48].

The fact that no physical difference among the proposed subpopulations of receptor proteins has yet been discerned [8], suggests an involvement of associated proteins. Steroid-bound PR that have not yet acquired the ability to bind to DNA have been found to exist in complexes containing hsp90 and p23 plus one of three immunophilins: hsp56, FKBP54 or CyP40 [51]. Depending on how each of these heterocomplexes are converted to the DNA binding form, this initial heterogeneity may be the source of the subsequent differential effects of salt on the kinetics of DNA binding. Alternatively, two populations of PR have been characterized in whole cells on the basis of the ability of geldampicin, which is closely related to geldanamycin, to prevent steroid binding [51]. Considerable functional heterogeneity has also been observed among glucocorticoid receptors, even though only one of the two receptor sequences has been described to bind steroid [52, 53]. The assembly of the steroid binding form of glucocorticoid receptors is thought to proceed via several intermediate complexes [54] and receptors that can not yet bind to DNA have been found to exist in complexes containing hsp90 and either hsp56, CyP40, or protein phosphatase 5 [55–57]. The large differences between the ability of RU 24,858 to inhibit dexamethasone induction of transcription, and dexamethasone inhibition of AP-1 induction, have been ascribed to separate receptor complexes [58]. Finally, two potential subpopulations of ER have been distinguished on the basis of their ability to bind to specific DNA sequences in both conventional ABCD [59] and filter binding [41] assays.

A recent detailed study of the DNA binding properties of the glucocorticoid receptor DNA binding domain has nicely documented that the dissociation of receptor fragments can be accelerated by added DNA. This behavior was observed for both monomer and dimers of the DNA-bound receptors and argues that the dissociation of DNA-bound complexes is not a simple diffusion of the receptor from DNA, since the rate of such a process would be unaffected by added DNA. The observed dependence of the dissociation rate on the concentration of added DNA is entirely consistent with two DNA binding sites per receptor that participate in intersegment transfer of receptor between two DNA molecules [24]. These data provide strong support for the mechanism of receptor localization on specific DNA sequences being through the faster intersegment transfer mechanism as opposed to other processes, such as linear searches [23]. The dissociation of full length PR from PRE in the ABCD assay was also accelerated dramatically by a 10-fold increase in soluble DNA [Fig. 2(c)]. Thus, PR may also undergo intersegment transfers due to the presence of two DNA binding sequences. It will be interesting to determine exactly where this second site is located and whether other steroid receptors are capable of intersegment transfer.

The current results were obtained, in large part, due to several advantages of the modified ABCD assay. First, in what is a major advantage of the modified ABCD assay over the conventional ABCD assay, the binding can be examined under equilibrium conditions. Furthermore, due to prior immobilization of the DNA by streptavidin–agarose, there is no delay between the end of receptor binding and the workup of the assay. Thus, the kinetics of binding (and dissociation) can be more readily examined. Second, the binding of receptor can be directly followed and easily quantified by scintillation counting. This is possible because the radioactive label is on the steroid bound to receptors as opposed to the DNA to which receptors, or other proteins, can bind. Third, sufficient quantities of receptor bind to the biotinylated DNA to allow the extracted receptors to be visualized by Western blotting (Fig. 6). This permits a direct and detailed analysis of bound receptors for differences in

molecular weight and/or charge. Fourth, the binding to DNA occurs with receptors in solution under conditions that can be adjusted to correlate with many *in vivo* processes.

In conclusion, the adventitious migration of $(NH_4)_2SO_4$ in the void volume of Sephadex G-10 chromatograms has uncovered a salt-induced increase in PR binding to specific DNA sequences, an increased selectivity of PRE binding by PR at moderate salt concentrations and a preferential binding of PR-A isoforms to a PRE. The same salt-induced increase occurred for PR binding to nonspecific DNA, which is biologically relevant as nonspecific DNA binding is a significant component of the orderly binding of proteins to specific sequences [22–24]. These effects of salt on the rate of DNA binding appear to reflect differential responses of subpopulations of receptors. As receptor binding to its cognate response element in regulated genes is a necessary event in steroid hormone action, heterogeneity at this step, coupled with the greater binding activity of PR-A vs PR-B, could provide additional mechanisms for differential control of gene expression in cells [60–62]. Whether this rate difference and specificity of PR subpopulation binding to DNA is decreased or increased both with other PRE sequences and in the context of chromatin will be important to determine.

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