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13. ABSTRACT *(Maximum 200*

The overall goal of the proposed research has been to understand the mechanisms by which breast cancers become resistant to hormone treatments. Specifically, the hormones involved are the steroidal agonists, estradiol and progesterone, the antagonists tamoxifen and RU-486, and the receptors to which these hormones bind, namely estrogen (ER) and progesterone receptors (PR). To this end, we initially used as models the PR of human breast cancer cells and the antiprogestin RU-486. We anticipated that the mechanisms we uncovered for progestins would be relevant to estrogens as well. This has turned out to be the case. We have discovered two proteins, which interact with antagonist-occupied receptors, and alter the direction of receptor-dependent transcription. 1. We isolated a coactivator, which we have dubbed L7/SPA, that interacts with tamoxifen-occupied ER or RU-486 occupied PR and **increases** transcription by 3-10 fold. It does not enhance agonist-dependent transcription, however. 2. We also isolated a corepressor protein that **suppresses** the partial agonist activity of tamoxifen or RU-486. These proteins may enhance (L7/SPA) or inhibit (corepressor) development of hormone resistant breast cancers, and this is now being tested in tumors taken from patients.

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K. B Hamilton 7/14/97

PI - Signature Date

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(5) INTRODUCTION

- The research in our laboratory focuses on breast cancer, and how the steroid hormones produced by the ovaries -- estradiol and progesterone -- are involved in the development and growth of these cancers.

- Additionally, because many breast cancers are hormone-dependent, which means that their growth is enhanced by estradiol and progesterone, treatment of the disease often involves the use of drugs that interfere with the actions of these hormones. Such interfering drugs are called steroid hormone antagonists. The best known of these antagonists are the antiestrogen, tamoxifen, and the antiprogestin, RU-486. Research in our laboratory seeks to understand just how steroid antagonists block the stimulatory effects of the steroid hormones in breast cancers.

- A key problem in the use of steroid antagonists to treat breast cancer, is that the tumors often respond well initially to the treatments and undergo remission. However, in time, the tumors acquire resistance to the antagonist treatment and resume growing. Research in our laboratory seeks to understand how tumors acquire resistance, with the goal of trying to block this process so that the effectiveness of antagonist treatments can be prolongued. Another outcome of this work is that it may suggest methods to design and screen for better antagonists; perhaps ones against which resistance will not so readily develop.

Therefore, the long-term goals of our research is to improve the strategies and outcomes of hormone therapies in breast cancers: by understanding how hormones control cancer growth, by understanding how tumors become resistant to hormone treatments, and by devising ways through which development of resistance can be avoided.

Estradiol and progesterone are hormonal agonists produced by the ovaries. These hormones then enter the blood stream and reach their target organs which, in addition to the breast, include the uterus and cervix, bones, blood vessels, skin, brain and other sites. These organs are "targets" for the hormones because their cell nuclei have proteins called "hormone receptors". When the hormone reaches the target cells, it passes through the cell cytoplasm and into the nucleus, where it encounters and binds the appropriate receptors. This binding activates the receptors, which in turn bind to specific DNA sequences located in front of the genes being regulated, and (usually) activates those genes. In other words, steroid hormone receptors are transcription factors whose function is controlled by hormone binding. Breast cancers whose growth is stimulated by estradiol and/or progesterone, do so because the tumor cells have estrogen- (ER) and/or progesterone receptors (PR) which bind these hormones. Like the agonists, the antagonists tamoxifen and RU-486, bind the tumor ER and PR respectively, and block the effects of the hormonal agonists at those sites; hence the term "antagonist" (1,2,3).

The structure of nuclear steroid receptors has been partially characterized. These are large proteins with modular functional domains. At the downstream, or C-terminal end, is the hormone binding domain (HBD). A hinge region separates the HBD from a centrally positioned DNA binding domain (DBD) through which the receptors interact with DNA.

Upstream of this, at the N-terminus, are transcriptional activation functions and other poorly defined domains. Both PR and ER have this same generic structure. There is an additional complexity with respect to PR however, in that there are two receptor isoforms that differ in size: PR B-receptors have a 164 amino-acid extension at the far N-terminus (the B-upstream segment, or BUS), which is missing in PR A-receptors. Because of this, the two PR isoforms have different gene regulatory properties when they are occupied by agonists or antagonists at the HBD (4,5).

We postulate that breast cancers become "resistant" to hormone therapy because antagonists acquire inappropriate, agonist-like, effects. The clinical consequences of such a functional switch are grave. The studies we proposed to address in this grant include the molecular mechanism by which antagonist-occupied progesterone B-receptors become transcriptional agonists.

The aims of the present work were to:

Aim 1. Characterize functional differences between A- and B-receptors in breast cancer cells. Band A-receptors are present together in breast cancers. Transient transfection methods show that antagonist-occupied B-receptors, but not A-receptors, can act like transcriptional agonists in a promoter-specific manner or when cAMP levels are increased. We plan to stably transfect PR-negative breast cancer cell lines with expression vectors encoding B- or A-receptors. This allows each receptor isoform to be studied independently while being expressed at normal levels in a physiological setting. The cells will be used to assess long-term growth effects, and transcriptional regulation, by progesterone agonists and antagonists, with or without increased cAMP levels. For transcriptional studies, different promoter-reporter constructs will be analyzed. These studies will define the effects of each receptor isoform on the functional end-points of transcription and cell proliferation in the physiologically relevant setting of breast cells.

Aim 2. BUS - The B-upstream segment. A third transcriptional activation domain unique to B-receptors? Since only PR B-receptors anomalously induce transcription in the presence of progesterone antagonists, we will focus on their unique 164 amino acid extension -- the B-upstream segment, or BUS. Preliminary data show that BUS contains a novel transcriptional activation function we call AF-3. This is in addition to two other AFs (AF-1 and AF-2) that are common to both receptor isoforms. We postulate that AF-3 functions by binding one or more coactivator proteins. We will construct a series of expression vectors of BUS alone, or of BUS fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of PR. These constructs will be tested for their ability to constitutively regulate transcription; to "squelch" full-length B-receptor actions; to complement A-receptors; and to be cAMP modulated, all in promoter and cell-specific contexts. Mutants will be constructed of 5 *ser-pro* clusters found on BUS in order to map AF-3, and to analyze the control of B-specific transcription by phosphorylation. The studies in this aim will functionally define and characterize AF-3; a site unique to B-receptors.

Aim 3. Mechanisms of AF-3 action in the BUS segment. Antagonist-occupied B-receptors activate transcription in a promoter-specific fashion: on the mouse mammary tumor virus

(MMTV) promoter when cAMP levels are elevated; on the *Herpes simplex* virus thymidine kinase (*tk*) promoter, through a novel, PRE-independent mechanism. First, we plan to identify cis-acting elements on the MMTV and *tk* promoters through which antagonist-occupied B-receptors stimulate transcription. Site-specific mutants of the MMTV promoter will seek the cis-acting elements that eliminate cAMP effects without loss of PR-regulated transcription. We will test the hypothesis that cAMP acts through novel DNA elements that cooperatively bind the ATF/CREB and HMG family of proteins, and interact with BUS. Linker-scanning mutants of the *tk* promoter will be used to define elements that mediate antagonist-occupied B-receptor stimulation of transcription. Second, protein-protein interactions between antagonist-occupied B-receptors and as yet unknown coactivators will be characterized using bacterially produced BUS fusion proteins, or the yeast two-hybrid system to identify, isolate and clone cDNAs encoding nuclear proteins that interact with BUS and to analyze their tissue-specific distribution. The studies in this aim will define novel coactivator proteins that interact with the B-receptor isoform of PR, and select their direction of transcription.

(6) BODY

In the 1996 Progress Report we reported that:

a) We have carried out an extensive series of studies related to Aim 1, using the newly constructed cell lines T47D-YA and T47D-YB, to explain the role of progestins on the growth of breast cancers. The details were described last year and the following paper has been extensively revised and is under review.

- *Steve Groshong, Gareth I. Owen, Bryn Grimison, Irene E. Schauer, Mary Daly, Tom Langan, Robert Sclafani, Carol Carter and Kathryn B. Horwitz. 1997. Biphasic regulation of breast cancer cell growth by progesterone: Role of the cdk inhibitors p21 and p27. Submitted.*

b) Our studies on PR phosphorylation, dealing with Aim 2 are published, and a reprint was appended in 1996.

- *Takimoto GS, Tung L, Hovland AR, Powell RL and Horwitz KB. Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. J Biol Chem 271:13308-13316, 1996.*

c) Other studies addressing Aim 2 were submitted for publication in 1996. The paper required revisions which have now been completed and the revised manuscript describing a novel inhibitory domain (IF) in PR has been resubmitted. The manuscript is appended.

- *Alicia R. Hovland, Roger L. Powell, Glenn S. Takimoto, Lin Tung and Kathryn B. Horwitz. 1997. A novel N-terminal inhibitory function (IF) and three activation functions (AFs) in human A- and B-receptors control their transcriptional diversity. Submitted.*

Drafts of the above three manuscripts were submitted in last year's progress report.

d) Protein-protein interaction screening for coactivators that bind to AF-3 was done, using a BUS-DBD construct (to satisfy requirements of Aim 3). We have discovered a novel protein complex that binds to the PR DBD and has strong DNA-dependent protein kinase activity. The results of these studies turn out to be related to the studies of Aim 1.

We have identified four HeLa cell nuclear proteins that bind to a glutathionine-S-transferase (GST)-human DBD_{PR} fusion protein, immobilized on a glutathionine-Sepharose 4B matrix. Microsequence and immunoblot analyses identify one of these proteins as the 113 kDa poly (ADP-ribose) polymerase (PARP). The other three DBD_{PR}-binding proteins are subunits of the DNA-dependent protein kinase (DNA-PK) holoenzyme: its DNA-binding regulatory heterodimer consisting of Ku70 and Ku86, and the 460 kDa catalytic subunit, $DNA\text{-}PK_{CS}$. The N-terminal 147 amino acids of the yeast transcription factor GAL4 containing its DBD, also copurify DNA-PK on the affinity matrix. However, neither a DBD_{PR} mutant lacking a structured first zinc finger, nor the wild-type DBD of estrogen receptors interacts with DNA-PK. The ability

of each fusion protein to "pull-down" DNA-PK on the affinity resin correlates precisely with the ability of the kinase -- while resin bound -- to autophosphorylate Ku70, Ku86 and DNA-PK$_{CS}$; to transphosphorylate the bait DBD; and to phosphorylate a DNA-PK-specific p53 peptide substrate. Surprisingly, DNase I treatment of bacterial extracts containing the fusion proteins, and of HeLa extracts containing the kinase, does not disrupt the association between DNA-PK and the DBDs, nor abolish its kinase activity. Similarly, addition of DNA, either with or without a PRE, does not further enhance the strong kinase activity seen in the absence of added DNA. Lastly, we find that catalytically active DNA-PK also copurifies with transcriptionally functional, FLAG-tagged, full-length human PR transiently expressed in HeLa cells. This suggests that the human PR/DNA-PK complex assembles *in vivo*. We propose that DNA-PK can assemble, and be brought to the proximity of a promoter through two mechanisms: direct binding to DNA as previously described, and direct binding to the DBDs of transcription factors, as described in this paper. Cooperativity between the two mechanisms may enhance the binding affinity and kinase activity of DNA-PK at a transcription complex, and may be one mechanism by which PR can regulate the activity of the transcription apparatus.

A paper describing these results has been submitted. The manuscript is appended.

- *C.A. Sartorius, G.S. Takimoto, J.K. Richer, L. Tung and K.B. Horwitz. Colocalization of poly(ADP-ribose) polymerase and the Ku autoantigen/DNA-dependent protein kinase holoenzyme on the DNA binding domain of progesterone receptors and regulation of phosphorylation in a DNA-independent manner. Submitted*

e) We have previously observed that under certain conditions, tamoxifen and RU-486 inappropriately activate gene transcription in a manner that resembles agonists -- that is, they behave as partial agonists. Such a switch in the activity of antagonists could be one mechanism by which resistance develops in a tumor. Thus if the antagonist treatment that is supposed to be inhibitory, becomes stimulatory instead, it is bad news for the patient. We undertook to explain this switching behavior, and speculated that in some tumors, receptors occupied by antagonists are capable of attracting a nuclear protein with "coactivator" properties, to the transcription complex. In the presence of such a coactivator, the transcription apparatus activates, rather than inhibits gene activity. To find such a putative coactivator we went fishing in a library of human proteins, using the PR hinge-HBD as bait. Because we were interested in proteins that bind PR in the presence of antagonists, we treated the hinge-HBD with RU-486. We isolated two interesting proteins that interact with RU-486 occupied PR and tamoxifen-occupied ER:

1. A coactivator that had not previously been described, which we call L7/SPA has precisely the properties we predicted. When we overexpress L7/SPA in breast tumor cells together with tamoxifen-occupied ER or RU-486 occupied PR, the antagonists have 3 to 10 times greater ability to activate a reporter gene than they do in the absence of L7/SPA. Thus L7/SPA strongly enhances the partial agonist transcriptional activity of these antagonists. **We predict that L7/SPA is overexpressed in hormone resistant tumors (ie in patients whose tumors resume growing despite treatment with tamoxifen).**

2. We isolated a second protein, N-CoR, with unanticipated "corepressor" properties. Its overexpression suppresses the partial agonist effects of tamoxifen and RU-486, and reverses the

excessive agonist effects produced by L7/SPA. **We predict that high levels of N-CoR expression can be used to identify tumors likely to respond well to treatment with antagonists.**

- *Twila A Jackson, Jennifer K Richer, David L Bain, Glenn S Takimoto, Lin Tung and Kathryn B Horwitz. 1997 The Partial Agonist Activity of Antagonist-Occupied Steroid Receptors is Controlled by a Novel Hinge Domain-Binding Coactivator L7/SPA and the Corepressors N-CoR and SMRT. Molecular Endocrinology 11: 693-705.*

The paper is appended.

(7) CONCLUSIONS

The findings described in *e)* address the overall goals of our research, and provide the first practical evidence that hormone resistance can be understood, and perhaps even be prevented.

To test the predictions made above, we are now analyzing a series of tumor pairs, taken from patients before tamoxifen treatment was started, and then again after the tumors became resistant to tamoxifen. In these pairs of tumors, we are measuring expression levels of L7/SPA, N-CoR and a related corepressor called SMRT. If our hypotheses are correct, the levels of the two corepressors will be high in the hormone responsive tumors at the start of treatment, and will fall in the resistant tumors coincident with a rise in L7/SPA. If so, we will have gained important insights into the mechanisms of hormone resistance. In future, by measuring the levels of these proteins we could identify tumors likely to respond well to antagonists, and could anticipate development of resistance and possibly block its onset.

Additionally, pharmaceutical companies are interested in using L7/SPA to screen new generations of antagonist ligands that they are developing. In theory, if an antagonist/receptor complex binds L7/SPA, we would predict that the antagonist in question can have partial agonist activity and would be more likely to elicit tumor resistance. Such a ligand would be discarded in favor of one that is a pure antagonist.

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5. Tung L, Mohamed KM, Hoeffler JP, Takimoto GS and **Horwitz KB.** Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements, and are dominantly inhibited by A-receptors. MOLEC ENDOCRINOL 7:1256-1265, 1993.

(9) APPENDIX

Three each:

Copies of manuscript submitted, Sartorius *et al*, 1997

Reprints of Jackson *et al*, *Mol Endocrinol* 11:693-705, 1997

The Partial Agonist Activity of Antagonist-Occupied Steroid Receptors Is Controlled by a Novel Hinge Domain-Binding Coactivator L7/SPA and the Corepressors N-CoR or SMRT

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Steroid receptor antagonists, such as the antiestrogen tamoxifen or the antiprogestin RU486, can have inappropriate agonist-like effects in tissues and tumors. To explain this paradox we postulated that coactivators are inadvertently brought to the promoters of DNA-bound, antagonist-occupied receptors. The human (h) progesterone receptor (PR) hinge-hormone binding domain (H-HBD) was used as bait in a two-hybrid screen of a HeLa cDNA library, in which the yeast cells were treated with RU486. We have isolated and characterized two interesting steroid receptor-interacting proteins that regulate transcription in opposite directions. The first is L7/SPA, a previously described 27-kDa protein containing a basic region leucine zipper domain, having no known nuclear function. When coexpressed with tamoxifen-occupied estrogen receptors (hER) or RU486-occupied hPR or glucocorticoid receptors (hGR), L7/SPA increases the partial agonist activity of the antagonists by 3- to 10-fold, but it has no effect on agonist-mediated transcription. The interaction of L7/SPA with hPR maps to the hinge region, and indeed, the hPR hinge region squelches L7/SPA-dependent induction of antagonist-mediated transcription. Interestingly, pure antagonists that lack partial agonist effects, such as the antiestrogen ICI164,384 or the antiprogestin ZK98299, cannot be up-regulated by L7/SPA. We also isolated, cloned, and sequenced the human homolog (hN-CoR) of the 270-kDa mouse (m) thyroid/retinoic acid receptor corepressor. Binding of hN-CoR maps to the hPR-HBD. mN-CoR, and a related human corepressor, SMRT, suppress RU486 or tamoxifen-mediated partial agonist activity by more than 90%. This suppression is completely squelched by overexpression of the hPR H-HBD. Additionally, both corepressors reverse the antagonist-dependent transcriptional up-regulation produced by L7/SPA. Our data suggest that the direction of transcription by antagonist-occupied steroid receptors can be controlled by the ratio of coactivators to corepressors recruited to the transcription complex by promoter-bound receptors. In normal tissues and in hormone-resistant breast cancers in which the agonist activity of mixed antagonists predominates, steroid receptors may be preferentially bound by coactivators. This suggests a strategy by which such partial agonist activity can be eliminated and by which candidate receptor ligands can be screened for this activity. (Molecular Endocrinology 11: 693–705, 1997)

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INTRODUCTION

Steroid hormone antagonists, such as the antiestrogen tamoxifen or the antiprogestin RU486, are synthetic pharmaceutical agents that have been found empirically to suppress the activity of natural steroidal agonists such as estradiol, progesterone, or glucocorticoids (1–3). The ability of antagonists to suppress the transcriptional effects of agonists has important clinical value (2, 4). However, in some tissues and tumors, instead of being inhibitory, steroid antagonists can have inappropriate, agonist-like effects (4–8). The precise mechanisms by which antagonists inhibit tran-

scription under some conditions, but stimulate it in others, are unknown (7).

Steroid hormones and their synthetic analogs bind to steroid receptors, which are members of a ligand-regulated family of nuclear transcription factors that includes, in addition to estrogen (ER) and progesterone (PR) receptors, the receptors for androgens, glucocorticoids (GR), and mineralocorticoids (9–11). These receptors belong to a distinct subgroup of the nuclear receptor superfamily, another subgroup of which includes the receptors for retinoic acids, vitamin D, and thyroid hormone (10). A key functional difference between steroid receptors and retinoic acid/thyroid hormone receptors is that the latter are constitutive transcriptional repressors, which bind to their cognate DNA-binding sites in the absence of ligand (12–14). In contrast, unliganded steroid receptors have little or no intrinsic DNA-binding ability or biological activity (12, 15). Instead, they require a ligand — either agonist or antagonist — to facilitate receptor-DNA interactions. The mechanisms by which unliganded retinoic acid/thyroid hormone receptors repress transcription were unknown until several recent studies described a new category of modulatory nuclear proteins having corepressor activity, which interact with the DNA-bound receptors and actively silence transcription (14, 16–22). Addition of ligand destabilizes corepressor binding to these receptors and activates transcription. These corepressors have been found to interact specifically only with unliganded members of the retinoic acid/thyroid hormone receptor subfamily, and they reportedly fail to interact with either unliganded or agonist-liganded members of the steroid receptor family (17, 19). No relationship has been known to exist between the mechanisms by which unliganded retinoic acid/thyroid hormone receptors repress transcription and the mechanisms by which antagonist-occupied steroid receptors inhibit the actions of agonists.

Little is known about the mechanisms by which steroid antagonists inappropriately activate transcription, although several models have been proposed. Partial agonist activity is often promoter- and cell type-specific (23, 24), and recently, a number of studies have shown that cross-talk between antagonist-occupied steroid receptors and cell surface-signaling pathways, such as activation by cAMP (4, 6, 25, 26), enhances these partial agonist effects, suggesting that unique receptor phosphorylation states mediate this activity.

We speculated that an alternative mechanism operates, namely that unique coactivator proteins are brought to the transcription complex by antagonist-occupied steroid receptors. To address this possibility, we used a LexA-human (h) PR hinge (H)-hormone binding domain (HBD) fusion protein as bait in a yeast two-hybrid screen of a HeLa cell cDNA library (27, 28). The assay incorporated a novel strategy in which the yeast cells were treated with the antiprogestin RU486. Using this screen, we have isolated two interesting proteins that regulate antagonist-occupied steroid receptors in opposite directions.

The first of these, L7 (29–36), is a 27-kDa cytoplasmic and nuclear protein believed to function in translational regulation (33) but of unknown nuclear function, which contains a canonical RNA- and DNA-binding leucine zipper bZIP dimerization domain (31). We find that human L7 or SPA (switch protein for antagonists) is a coactivator that strongly enhances transcription of RU486-occupied hPR or hGR, and tamoxifen-occupied hER, but has no effect on agonist-dependent transcription by these receptors. The second isolate is the human homolog (hN-CoR) of the mouse (m) retinoic acid/thyroid hormone receptor corepressor, N-CoR (17). Both mN-CoR and the related human corepressor SMRT (silencing mediator for retinoid and thyroid hormone receptors) (19), suppress the agonist-like transcriptional activity of RU486-occupied hPR and tamoxifen-occupied hER. Transcriptional repression mediated by N-CoR is reversed by L7/SPA.

We propose that the inhibitory pharmacological effects of antagonist-occupied steroid receptors involves the adventitious recruitment of a transcriptional corepressor that has no normal physiological function in steroid hormone action, whereas the partial agonist-stimulatory effects of antagonists involves recruitment of novel coactivators. Thus, the ratio of corepressors to coactivators that are bound to the transcription complex through the antagonist-occupied receptors determines whether the outcome is inhibitory or stimulatory. This property of antagonist-occupied receptors has therapeutic implications and suggests methods by which candidate receptor ligands can be screened for their partial *vs.* pure antagonist pharmacology.

RESULTS

Yeast Two-Hybrid Screening Strategy

To isolate proteins that interact with antagonist-occupied steroid receptors, we first asked whether agonists and antagonists have the appropriate transcriptional responses in yeast cells (Fig. 1A). L40 cells were transformed with a fragment of hPR (37, 38) consisting of the hinge region (H) and hormone binding domain (HBD) (amino acids 638–933) fused to LexA (pLexA: H-HBD). The cells were then treated with 1 μM progestin R5020, or 10 μM type II antiprogestins RU486 or ZK112993, or the type I antiprogestin ZK98299 as shown. R5020 strongly activates β-galactosidase transcription from the LexA operator, whereas the antagonists alone have no effect. However, RU486 and ZK112993 completely abolish R5020-dependent transcription, while ZK98299, which has a lower binding affinity for hPR, was 85% inhibitory. We conclude that appropriate antiprogestin-regulated transcriptional inhibition can be elicited in yeast cells.

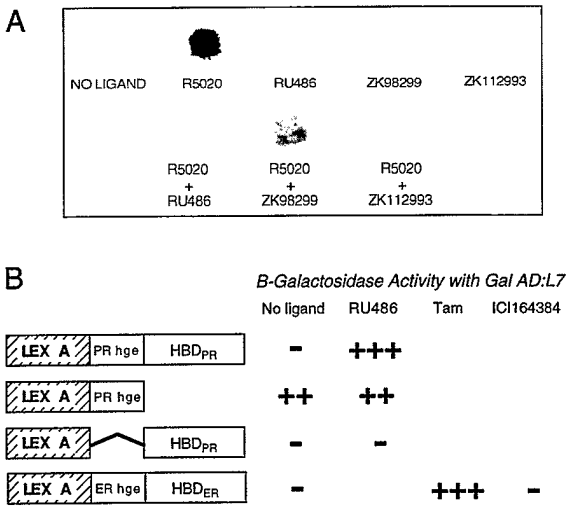

Fig. 1. L7/SPA, a Novel Protein That Interacts with Antagonist-Occupied Steroid Receptors

A, Antiprogestins can inhibit transcription by R5020 in yeast. The yeast two-hybrid strain L40 carrying a LexA promoter-LacZ reporter was cotransformed with an expression vector encoding the hPR hinge and hormone binding domain (hge, HBD) fused to LexA. Yeast cells were treated 48 h at 30 C with 1 μM of the agonist R5020, 10 μM of the three antiprogestins shown, either alone or in combination. Yeast colonies were lifted on a nitrocellulose filter and lysed, and a β-galactosidase assay was performed. B, The L7/SPA interaction maps to the hPR hinge region. The yeast two-hybrid strain L40 was cotransformed with the hPR or hER expression vectors encoding the fusion proteins shown in the figure and the vector encoding the Gal4 activation domain:L7/SPA fusion protein recovered from the library. Transformed yeast cells were grown for 2 days at 30 C on media containing 10 μM of the indicated antagonists. Colonies were lifted on a nitrocellulose filter and lysed, and a β-galactosidase assay was performed.

To identify proteins that mediate the agonist and/or antagonist activity of antiprogestins, pLexA:H-HBD was then used as bait to screen a HeLa cell cDNA library in yeast cells exposed to 10 μM RU486. Approximately 10 million recombinants were screened, of which 28 clones, identified as unique by restriction mapping, were classified as positive in preliminary assays.

The Coactivator, L7/SPA

One clone, TJ48, interacted with the hPR H-HBD but not with a lamin bait, and had no intrinsic transcriptional activity in the GAL4 AD library vector (data not shown). TJ48 was sequenced and found to be identical to nucleotide (nt) 54 to 744 of the L7/SPA (35) cDNA, which encodes a 27-kDa protein originally defined as a potent autoantigen associated with the large ribosomal subunit (32, 34).The N terminus of L7/SPA contains a basic region leucine zipper (bZIP) domain (39, 40), through which it forms stable homodimers that bind to RNA and double-stranded DNA (31, 35). The protein is detectable in the cytoplasm and nuclei but not nucleoli of human cell lines (34), and the transcript is expressed in a variety of adult mouse tissues and in human T47D and HeLa cell lines (data not shown). It has no known nuclear function.

Full-length L7/SPA cDNA was isolated by RT-PCR from HeLa cell RNA and cloned into the pGEX 4T1 glutathione-S-transferase (GST) plasmid and into the pSG5 mammalian expression vector. The interaction between L7/SPA and the hPR H-HBD was confirmed by GST pull-down (data not shown) of *in vitro* translated L7/SPA. To further map the L7/SPA-hPR interaction, hPR H-HBD, H, or HBD/LexA bait fusion proteins were expressed in yeast cells together with the original GAL4 AD-L7/SPA library fusion protein, which lacks 18 N-terminal amino acids. The cells were treated or not with RU486, and β-galactosidase activity driven by the LexA promoter was measured (Fig. 1B). As shown, transcriptional activity in the presence of H-HBD is dependent on treatment of the cells with RU486. β-Galactosidase activity is entirely absent, however, in the presence of HBD alone, regardless of hormone treatment, and is constitutively active in the presence of the hinge domain. This suggests that L7/SPA binds to H, but that it is ordinarily blocked by the HBD, and that this inhibition can be relieved by RU486 occupancy of the HBD. Similarly, L7/SPA binding to the H-HBD of hER is dependent on occupancy by the antiestrogen tamoxifen, whereas the pure antiestrogen, ICI 164,384 (3), does not promote interactions between L7/SPA and hER.

To test the effect, if any, of L7/SPA on steroid receptor-mediated transcription in mammalian cells, a PRE_2-$TATA_{tk}$-CAT reporter was transfected into HeLa cells, and dexamethasone (Dex) or RU486-regulated transcription from the endogenous GR was measured (Fig. 2) in the absence or presence of exogenous full-length L7/SPA. Dexamethasone strongly induces transcription (lane 2) which is unaltered by overexpression of L7/SPA (lane 3). RU486 behaves as a partial agonist under these conditions (compare lanes 1 and 4). Surprisingly, the agonist activity of RU486 is enhanced 10-fold by overexpression of L7/SPA (lane 5), and this extensive up-regulation can be completely squelched by the hPR hinge domain (lane 6). Similar results are observed in HeLa cells transiently overexpressing recombinant hGR (lanes 7–12). Thus, L7/SPA appears to have the astonishing ability to strongly enhance the partial agonist activity of a steroid antagonist, without altering agonist-dependent transcription.

RU486 is a type II antiglucocorticoid/antiprogestin that promotes receptor-DNA interactions (41, 42) and has partial agonist activity. Type I antagonists, such as the antiprogestin ZK98299, lack this activity (42, 43). The effect of L7/SPA on ZK98299-regulated transcription was tested using a construct consisting of a truncated hPR (DBD-H-HBD), lacking the N terminus. As shown in Fig. 3A, the hPR C terminus (or DBD-H-HBD) is strongly activated by R5020 (lanes 3 and 4) which is not modified by L7/SPA (lane 4). On the other hand,

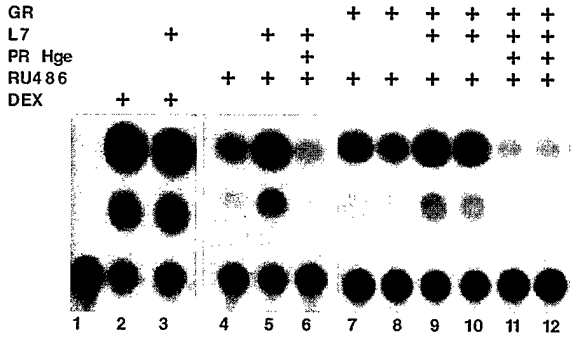

Fig. 2. L7/SPA Enhances the Partial Agonist Activity of RU486- but Not Dexamethasone-Mediated hGR Transcriptional Activity and This Activity Is Squelched by the hPR Hinge Region

HeLa cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, with (lanes 7–12) or without (lanes 1–6) 10 ng hGR expression vector, and 5 μg full-length L7/SPA, plus or minus 5 μg hPR hinge expression vector as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 100 nM RU486 or 10 nM dexamethasone for another 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC.

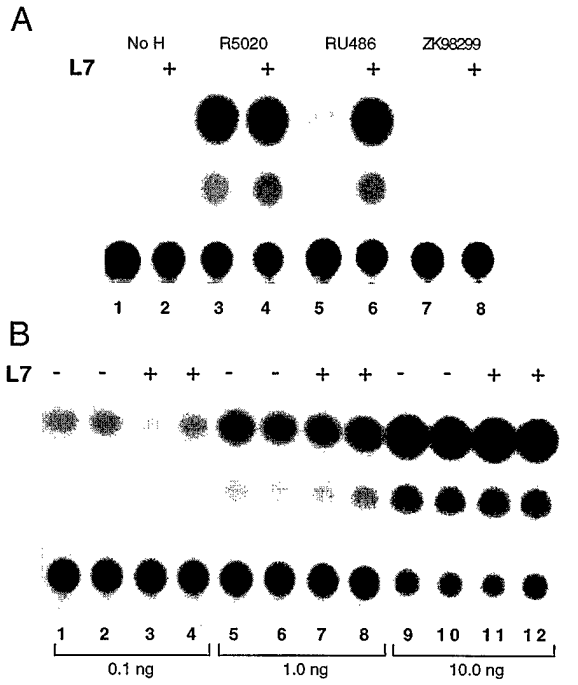

Fig. 3. L7/SPA Stimulates the Transcriptional Activity of the RU486-Occupied but Not the ZK98299- or R5020-Occupied hPR

A, HeLa cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, 10 ng hPR C-terminus consisting of the DNA binding domain linked to the hinge region and HBD, and 5 μg full-length L7/SPA expression vectors as indicated in the figure (+). Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 100 nM RU486 or ZK98299 or 10 nM R5020. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC. B, HeLa cells were transfected as above with 0.1, 1.0, and 10 ng full-length hPR B-receptors and treated with 10 nM R5020.

the partial agonist activity of RU486 (lane 5) on the PR C terminus is strongly enhanced by L7/SPA (compare lanes 5 and 6). In contrast, ZK98299 lacks partial agonist activity (lane 7) and is unaffected by L7/SPA (lane 8). Full-length hPR B-receptors have the same properties (not shown) as do hPR specificity mutants (43) in which the PRE-binding specificity of the PR DBD has been switched to an estrogen response element by mutation of three key amino acids (44, 45) in the first zinc finger (data not shown). This was done to rule out any effects of endogenous GR, on recombinant hPR-regulated transcription.

The data in Fig. 3A show that L7/SPA influences antagonist- but not agonist-mediated transcription. To analyze its effects on the agonist in more detail, submaximal concentrations of hPR B-receptors were tested (Fig. 3B). HeLa cells were transfected with the reporter, with 0.1, 1.0, and 10.0 ng of the B-receptor expression vector in the presence or absence of the L7/SPA expression vector, and the cells were treated with saturating concentrations of R5020. At 0.1 and 1.0 ng DNA, B receptor expression and its ability to transactivate PRE_2-$TATA_{tk}$-CAT are submaximal, yet L7/SPA is unable to enhance transcription. These data suggest that the effects of L7/SPA are indeed antagonist-specific.

Depending on their structure, some antiestrogens (tamoxifen, for example) possess partial agonist activity, whereas other antiestrogens (such as ICI 164,384) do not (3). To determine whether L7/SPA modifies the effects of antiestrogens, HeLa cells were transfected with wild type hER and the ERE_2-$TATA_{tk}$-CAT reporter in the presence or absence of the L7/SPA expression vector and were either left untreated or treated with 17β-estradiol (Fig. 4A) or the antiestrogens shown (Fig. 4B). Estradiol-dependent hER-mediated transcription is not influenced by L7/SPA even under submaximal hER expression levels (Fig. 4A). In contrast, the partial agonist activity of tamoxifen is further enhanced by L7/SPA overexpression (Fig. 4B). This increase can be squelched (46) by expression of the hPR hinge region. The extent of L7/SPA squelching by the hPR hinge can not be gauged without extensive titration studies, but we find that hPR hinge overexpression can reduce the partial agonist effect of tamoxifen even in the absence of L7/SPA (Fig. 4B), suggesting that endogenous cellular coactivators can also bind the hinge domain. On the other hand, like ZK98299, the antiestrogen ICI 164,384, which lacks partial agonist activity, is unaffected by L7/SPA. Thus, the activity of steroid antagonists that have partial agonist activity can be further enhanced by expression of L7/SPA, while pure antagonists and agonists are unaffected by this unusual coactivator. This explains the failure of ICI 164,384 to promote hER interaction with L7/SPA in the yeast two-hybrid screen (Fig. 1B).

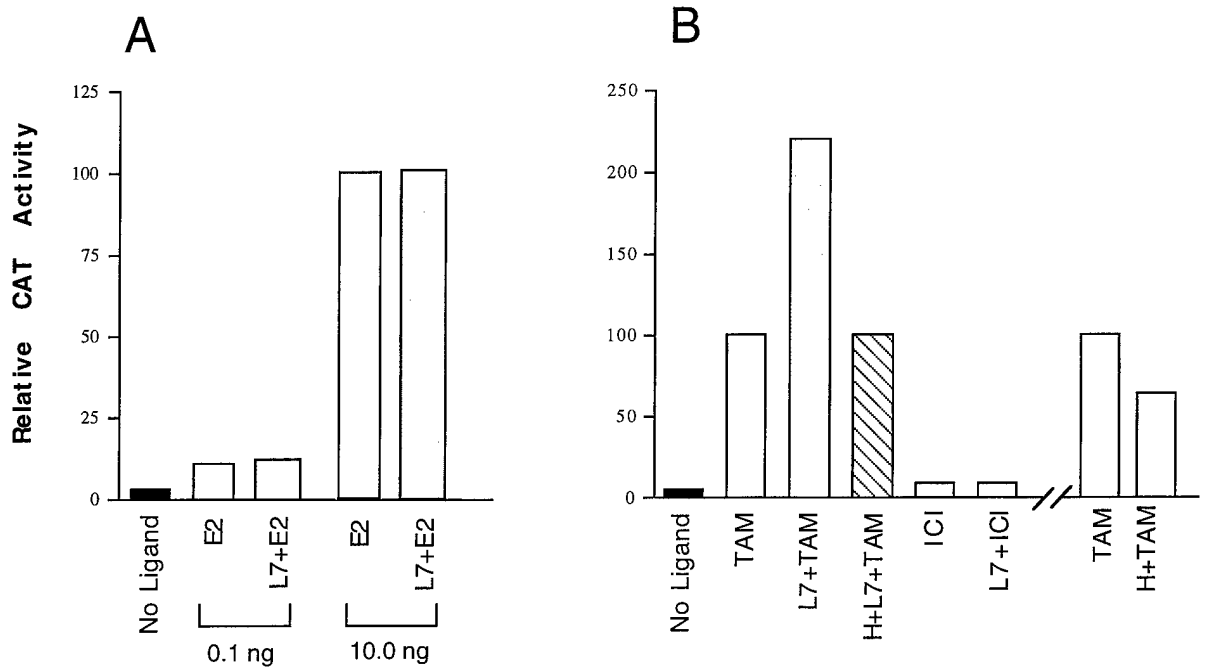

Fig. 4. L7/SPA Enhances the Partial Agonist Effects of Tamoxifen but Has No Effect on Estradiol or the Pure Antagonist ICI 164,384

A, HeLa cells were cotransfected with 2 μg ERE_2-$TATA_{tk}$-CAT reporter and 0.1 or 10 ng hER with or without 5 μg L7/SPA as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were either untreated or treated with 10 nM 17β-estradiol for another 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). B, HeLa cells were transfected as above, in the presence or absence of 5 μg hPR hinge (H) expression vector as indicated, and the cells were either untreated or treated with 100 nM tamoxifen or ICI 164,384. In panel A, estradiol-mediated CAT activity was set at 100% and in panel B, tamoxifen-mediated CAT activity was set at 100%. The agonist activity of tamoxifen is 50% that of estradiol.

The Corepressors N-CoR and SMRT

A second cDNA clone, TJ53, was isolated by yeast two-hybrid screening using RU486-occupied hPR H-HBD as bait (Fig. 5). Sequence analysis showed that this 3300-nt human cDNA (hN-CoR ID) was homologous to RIP13 (21), which is the interaction domain (ID) and surrounding C-terminal sequences of the 270-kDa mouse nuclear receptor corepressor (mN-CoR). Mouse N-CoR mediates ligand-independent repression by the thyroid hormone/retinoic acid receptor subfamily (17). Treatment with thyroid hormone or retinoic acid dissociates mN-CoR from the cognate receptors. It is noteworthy that the evidence presented in these studies indicates that mN-CoR does not interact with steroid receptors.

Using the hN-CoR ID cDNA recovered from the HeLa cell library cloned into the yeast vector, a HeLa cell cDNA cell library cloned into a bacterial vector was screened and, together with RT-PCR of HeLa and T47D cell RNA, the entire human coding sequence was obtained, sequenced, and compared with the murine protein-coding sequence (Fig. 5). In addition to a 7359-nt open reading frame that predicts a 2453-amino acid protein (hN-CoR1), two apparent N-terminal splice variants that result in loss of amino acids 83–206 (hN-CoR2) and amino acids 83–147 (hN-CoR3) in the N-terminal repressor domain (17) have been detected for the human corepressor. The amino acid identity between mN-CoR and hN-CoR1 is high (98.91%), with the greatest divergence observed in the second repressor domain (RD2) in which the identity falls to 80.4%. Because of this identity, we have used mN-CoR in most studies described below.

We find that the hN-CoR ID interacts with and modulates the activity of RU486-occupied hPR H-HBD and tamoxifen-occupied hER H-HBD. As shown in Fig. 6, the LexA/hPR fusion bait proteins were coexpressed in yeast cells together with the GAL4-AD/hN-CoR ID in the absence or presence of three antiprogestins. β-Galactosidase activity was dependent on the presence of RU486 when either the H-HBD or the HBD constructs were present in the cells, but it was uninducible with the H construct, suggesting that the hN-CoR ID interacts with the hPR HBD but not the hinge domain. This interaction is promoted by RU486 and by another type II antiprogestin, ZK112993, but not by the type I antiprogestin ZK98299. The latter is a pure antagonist that appears to inhibit hPR interactions with DNA (42, 47). Similarly, the interaction between the hER H-HBD and hN-CoR ID is very strong with tamoxifen occupancy, but minimal with the pure antiestrogen, ICI 164,384.

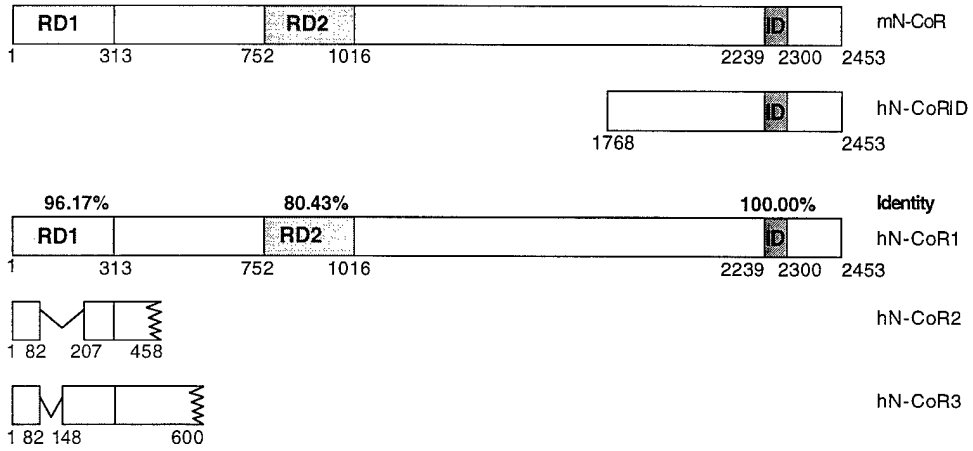

Fig. 5. A Comparison of the Mouse and Human N-CoR

The mN-CoR, as reported by Hörlein *et al.* (17) is shown at the top including two repressor domains (RD) at the N terminus and an interaction domain (ID) at the C terminus. The HeLa cell library clone isolated by yeast two-hybrid screen, TJ53, is referred to herein as hN-CoR ID. Also shown is the full-length hN-CoR1, together with its percent amino acid identity to mN-CoR. Two other clones contain deletions in RD1: hN-CoR2, an independent isolate from the HeLa cDNA bacterial library, lacks amino acids 83–206; hN-CoR3, an expressed tag sequence (Genbank accession number N33258), lacks amino acids 83–147. The amino acid sequence and protein structure downstream of amino acid 458 for hN-CoR2 and amino acid 600 for hN-CoR3 are unknown. Numbers refer to amino acids.

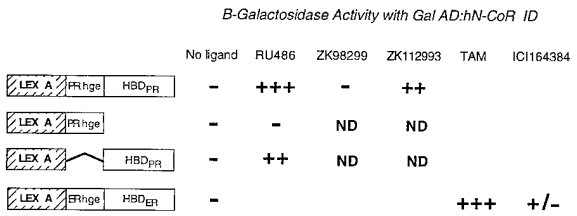

Fig. 6. The hN-CoR ID Interacts with the HBD of Steroid Receptors Occupied by Antagonists That Have Partial Agonist Activity, but Not to Pure Antagonists

The yeast two-hybrid strain L40 was cotransformed with the hPR or hER expression vectors encoding the fusion proteins shown in the figure and the vector encoding the Gal4 activation domain:hN-CoR ID fusion protein recovered from the library. Transformed yeast cells were grown for 2 days at 30 C on media containing no hormone addition or 100 μM of the indicated antagonists. Colonies were lifted on a nitrocellulose filter and lysed and a β-galactosidase assay was performed.

To characterize the function of the full-length corepressor on steroid receptors, we used mN-CoR (17) and the related corepressor SMRT, a 168-kDa protein described by Chen and Evans (19), having 48% identity to RIP13 (21), the C terminus of N-CoR. Similar to mN-CoR, the inhibitory properties of SMRT are restricted to the unliganded thyroid hormone/retinoic acid receptors. Figure 7 shows that either mN-CoR or SMRT suppresses the partial agonist activity of steroid antagonists. COS cells were transfected with the full-length hPR or hER expression vectors, expression vectors for mN-CoR or SMRT, the appropriate chloramphenicol acetyl transferase (CAT) reporter, and treated with RU486 or tamoxifen. As shown, the partial agonist activity of both steroid antagonists was more than 90% suppressed by either corepressor. This repression by mN-CoR or SMRT of the partial agonist effect of tamoxifen can be entirely squelched by coexpression of the hPR H-HBD (Fig. 8). Figure 8 also shows that the hPR H-HBD alone partially suppresses the agonist activity of tamoxifen, indicating that, in the absence of overexpressed N-CoR, other factors, possibly coactivators, may bind to the H-HBD.

Both N-CoR and SMRT are large proteins whose functional domains have not yet been well characterized. We find, for example, that the hN-CoR ID contains a transcriptional activation function that localizes to the C-terminal 240 amino acids (data not shown). Thus, these proteins may have functions other than the repressor ones described previously (17, 19). We also find (data not shown) that mN-CoR subtly suppresses, while SMRT subtly increases, the level of basal transcription from the promoters used in the present studies. This difference may be reflected in the data shown in Fig. 9, which show subtle effects of the corepressors on steroid receptor agonist-dependent transcription. In this study COS cells were transfected with expression vectors for hER, hPR A-receptors, and hPR B-receptors lacking 42 C-terminal amino acids (BΔ42). This truncation converts RU486 into an agonist (48). Cells were treated with the appropriate agonists (or RU486 in the case of BΔ42) in the presence or absence of SMRT or mN-CoR. We find that under these conditions of agonist-dependent transcription, SMRT consistently slightly up-regulates transcription (which rises to 3-fold in the case of BΔ42 and RU486), whereas mN-CoR consistently slightly decreases transcrip-

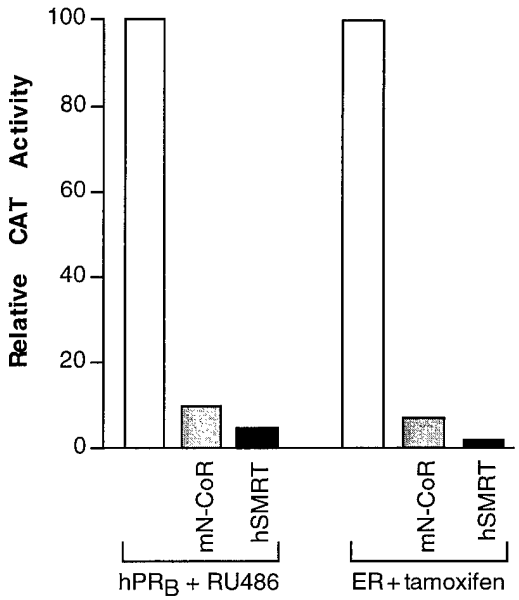

Fig. 7. Mouse N-CoR and Human SMRT Repress the Partial Agonist Activity of RU486-Occupied hPRB and Tamoxifen-Occupied hER

COS cells were cotransfected with 2 μg PRE$_2$-TATA$_{tk}$-CAT or ERE$_2$-TATA$_{tk}$-CAT reporters, 10 ng hPRB or hER, and 5 μg mN-CoR or hSMRT expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and cells were either untreated or treated with 100 nM RU486 or tamoxifen. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by phosphorimaging setting the partial agonist activity of RU486 or tamoxifen (*open bars*) at 100%.

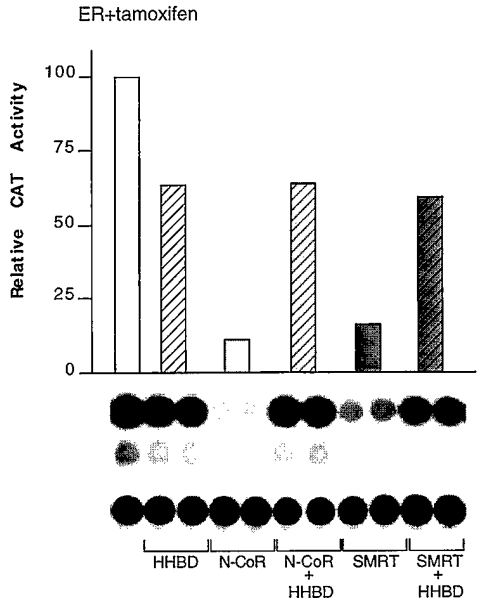

Fig. 8. Mouse N-CoR and hSMRT Suppress the Partial Agonist Activity of Tamoxifen-Occupied hER and the Effect of These Corepressors Is Squelched by the hPR H-HBD

COS cells were cotransfected with 2 μg ERE$_2$-TATA$_{tk}$-CAT reporter, 10 ng HEGO with or without 5 μg mN-CoR or hSMRT, and 5 μg hPR H-HBD expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were treated with 100 nM tamoxifen for 24 h. Cell lysates were normalized to β-galactosidase activity, CAT assays were performed by TLC, and duplicate experimental points were quantitated by phosphorimaging and averaged. The partial agonist activity of tamoxifen-occupied hER was set at 100% (*open bar*). In this study, tamoxifen had 47% the agonist activity of estradiol (not shown).

tion. These subtle effects of the corepressors with agonists are in sharp contrast to the major inhibitory effects of both corepressors with antagonists (see Figs. 7 and 8). We ascribe these agonist-dependent effects of the corepressors to the influence that they appear to have on basal transcription levels of the promoters used in the present studies, but other explanations are possible. In contrast, L7/SPA has no effect on basal transcription levels and modulates only antagonist-dependent transcription.

The L7/SPA Coactivator Plus the Corepressors?

Because coactivators and corepressors appear to "coexist" in cells, it seems reasonable to propose that the ultimate direction of transcription under control of antagonist-occupied steroid receptors depends on the relative cellular levels of these two, offsetting classes of coregulatory proteins. The preliminary study in Fig. 10 suggests that this is indeed the case. Here, HeLa cells were transfected with an hGR expression vector alone, or together with L7/SPA or SMRT (either alone or in combination), and the cells were treated with RU486. As shown, L7/SPA alone enhances RU486-dependent transcription from the PRE$_2$-TATA$_{tk}$-CAT reporter, SMRT alone strongly inhibits it, but when present together, one coregulator neutralizes the effects of the other.

DISCUSSION

Antagonist-Mediated Transcriptional Inhibition

Steroid receptor antagonists are pharmacological agents that have been synthesized and selected for clinical use on the basis of empirical tests that show that they inhibit the actions of the cognate natural hormonal agonists. Although there is speculation about the existence of natural antagonists, none have yet been described. The mechanisms by which synthetic antagonists inhibit transcription of steroid hormone-regulated genes have been intensively studied. Antagonists compete with agonists for binding to the HBD of the receptors and then prevent or modify receptor dimerization or DNA binding, or facilitate receptor-DNA interactions but form a transcriptionally nonproductive complex (1, 24, 41, 42). These scenarios confer a passive role on the inhibitory effects of

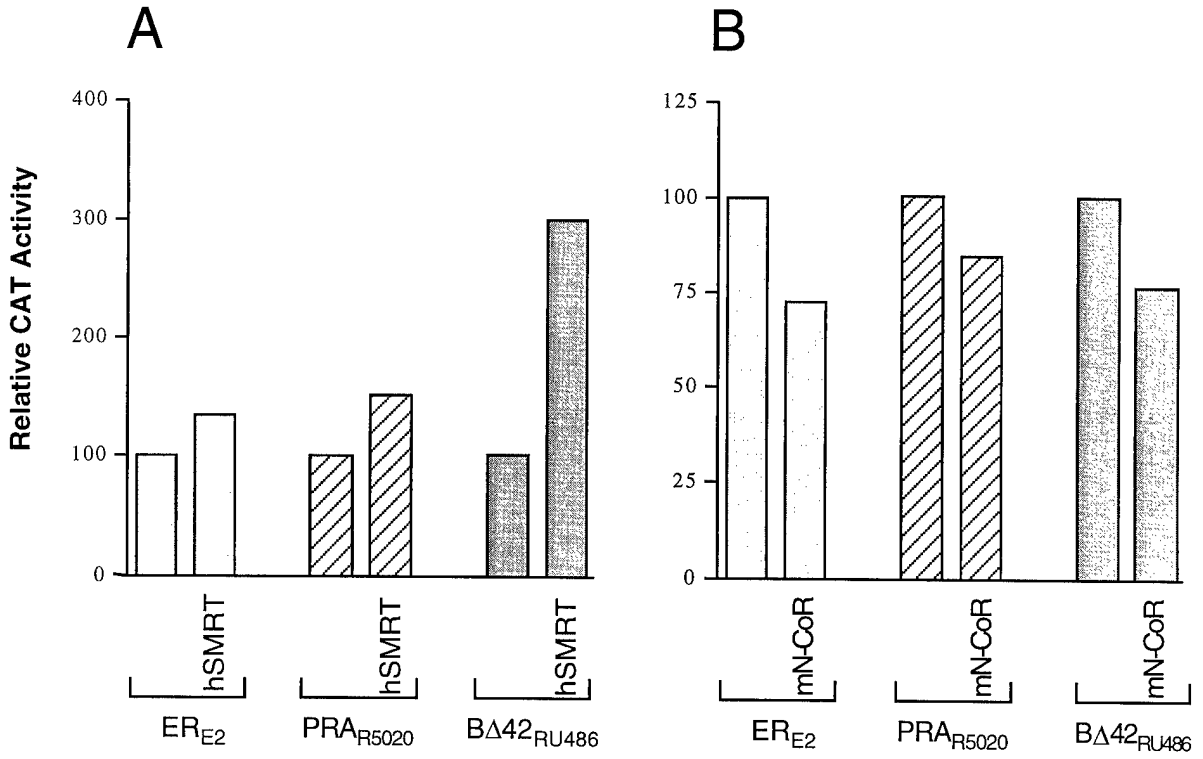

Fig. 9. The Effects of mN-CoR and hSMRT on Agonist-Mediated Transcription

COS cells were cotransfected with 2 μg PRE$_2$-TATA$_{tk}$-CAT or ERE$_2$-TATA$_{tk}$-CAT reporters, 10 ng hER, hPRA, or hPRBΔ42 expression vectors, along with 5 μg hSMRT (A) or mN-CoR (B) expression vectors as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and cells were either untreated or treated with 10 nM of the hormones shown. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantitated by phosphorimaging. *Bars* represent the average of duplicate experimental points. Transcription in the absence of corepressors was set at 100%.

antagonists, in which they simply compete for agonist binding, and therefore block agonist actions.

However, the data presented herein suggest that steroid antagonists can also actively repress transcription. We show that antagonists are able to do so, by recruiting to DNA-bound steroid receptors one or more endogenous corepressors whose normal cellular function is to mediate gene repression by unrelated transcriptional repressors, such as the unliganded retinoic acid/thyroid hormone receptors. We speculate that these corepressors have no normal function with respect to agonist action, but that they are adventitiously brought to promoter-bound steroid receptors, when they are occupied by synthetic antagonists. McDonnell *et al.* (8) and others (48–50) have proposed that agonists and antagonists stabilize different conformational states of steroid receptors. If so, it is possible that a subset of synthetic antagonists freeze DNA-bound steroid receptors in a unique conformational state that enhances the binding affinity of corepressors for the HBD of the receptors. Furthermore, it is possible that on promoters in which steroid receptors repress transcription (16), recruitment of corepressors comes into play.

We have taken advantage of the partial agonist property of RU486 and tamoxifen to demonstrate the recruitment of corepressors to steroid receptors and the resultant transcriptional inhibition. On the other hand, steroids such as ZK98299 (Fig. 1) and ICI 164,384 are also competent antagonists, yet our data suggest that they do not promote receptor-corepressor interactions. It is possible that two, quite different, mechanisms are involved in antagonist-dependent transcriptional inhibition: type I inhibitors may function passively by sequestering the receptors away from the transcription complex, whereas type II inhibitors, which foster receptor binding to DNA, function actively by recruiting corepressors that block transcription. However, no definitive mechanism can be advanced until the controversy surrounding the DNA binding properties, or lack thereof, of antagonist-occupied receptors is resolved.

Recruitment of corepressors explains another physiological puzzle, *i.e.* the ability of some steroid antagonists to suppress gene transcription even in the absence of a hormonal agonist (51). As described above, current assumptions hold that antagonist-occupied receptors suppress agonist-regulated transcription by competitive inhibition. Our model predicts that a gene that contains a steroid hormone response element, but is up-regulated by any signal, including a nonsteroidal one, can be suppressed by recruitment of a corepressor to antagonist-occupied complexes

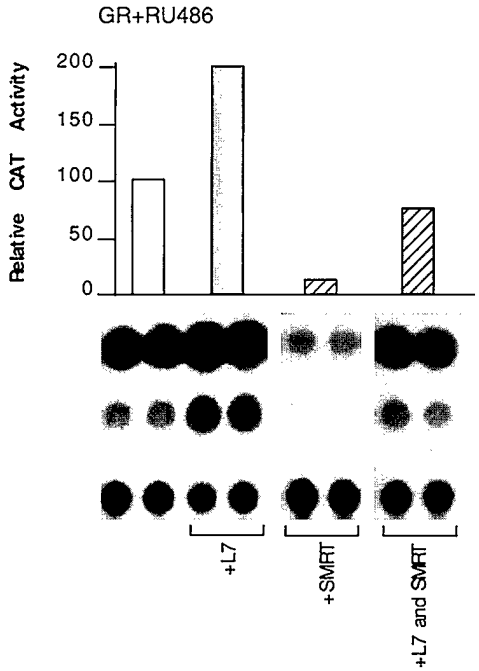

Fig. 10. The Partial Agonist Activity of RU486-Occupied hGR Is Controlled by the Ratio of L7/SPA to SMRT Recruited to the Transcription Complex

COS cells were cotransfected with 2 μg PRE_2-$TATA_{tk}$-CAT reporter, with 10 ng hGR, and with 5 μg L7/SPA or hSMRT expression vectors alone or in combination as indicated in the figure. Twenty-four hours after transfection, the medium was changed, and the cells were treated with 100 nM RU486 for 24 h. Cell lysates were normalized to β-galactosidase activity, CAT assays were performed by TLC, and duplicate experimental points were quantitated by phosphorimaging and averaged. The partial agonist activity of RU486-occupied hGR was set at 100% (*open bar*).

bound to the hormone response element of that gene, leading to inhibition in the absence of an agonist.

Partial Agonists and the Coactivator, L7/SPA

When steroid antagonists are used therapeutically, two problems commonly arise. The first is that the drug may have the desired effect in one tissue, but the opposite effect in another. Tamoxifen is a case in point. It is appropriately antiestrogenic in the breast but acts like an estrogen in the uterus, where it induces endometrial cancers (52–56). The mechanisms underlying these undesirable tissue-specific agonist effects are unclear. The second problem arises in tamoxifen-responsive breast cancers, which not only acquire resistance to tamoxifen treatment after a period of time, but in which tamoxifen actively switches to an agonist (7). We have speculated that the mechanisms involved in tissue-specific agonist effects of antagonists, and in the acquired resistance of tumors to tamoxifen treatment, are similar and that both are mediated by coactivators recruited to the transcription complex by the antagonists. Note that tamoxifen-resistant tumors often respond to second-line treatment with a pure antiestrogen or other hormone therapies (57–59), underscoring our contention that pure antagonists operate through mechanisms that differ from those of antagonists with partial agonist activity.

We have now isolated a protein, L7/SPA (29–36), that distinguishes between these two classes of steroid antagonists. In the cytoplasm, L7/SPA associates with the large ribosomal subunit (30), where it inhibits cell-free translation (33). Like other ribosomal proteins, it is a potent autoantigen (32, 34). However, L7/SPA is also an extranucleolar nuclear protein of unknown function (34). Recently an α-helical leucine zipper domain (bZIP) was mapped to the N-terminal 15–49 amino acids of the 248-amino acid protein, through which it homodimerizes and binds to DNA and RNA (31, 35).

We isolated L7/SPA by its ability to bind the H-HBD of hPR, mapped that binding to the hPR hinge region, and showed that L7/SPA strongly enhances transcription by antagonist-occupied hGR, hER, and hPR, but interestingly, that it has no effect on agonist-mediated transcription. L7/SPA therefore exhibits the novel property of being an antagonist-specific transcriptional coactivator whose binding maps to the hinge region. This is the first description, to our knowledge, of an activation function in this region, although an inhibitory function has previously been described (60). Moreover, YL8A (36), the *Saccharomyces cerevisiae* homolog of mammalian L7/SPA, lacks the canonical N-terminal bZIP domain, but the remainder of the molecule shares 56% amino acid identity and 81% conservation with the human protein. As we show in Fig. 1A, RU486 has no partial agonist activity in yeast, suggesting perhaps that the bZIP domain of L7/SPA is important for its coactivator activity, and studies to address this hypothesis are in progress.

There are multiple examples, particularly with tamoxifen, demonstrating agonist activity of antagonists. Several groups have shown that tamoxifen agonism is especially strong on unusual EREs, including the raloxifene response element (61), AP-1 sites (62), and cooperating weak EREs (63). It is interesting to speculate that L7/SPA might be a very potent coactivator at such elements.

Steroid Antagonists and the Combined Effects of Coregulators

The present studies show that antagonist-occupied steroid receptors are targets for the actions of both corepressors and coactivators. It seems logical to suppose that the sum of the combined effects of these coregulatory proteins, determined by their relative cellular concentrations and binding affinities for the receptors, will control the direction of transcription by a particular ligand. This model predicts that the inhibitory or stimulatory efficacy of an antagonist will vary among tissues and tumors depending on the levels

and availability of the endogenous coregulators, and suggests that by modulating those levels, it may be possible to control the direction of transcription by the antagonist. Moreover, if the ability to bind an antagonist-specific coactivator is the mark of an antagonist having partial agonist activity, this property should be useful for the pharmacological screening of candidate ligands.

MATERIALS AND METHODS

Plasmid Construction

The hPR H-HBD and hinge region (H or hge) alone, including the complete endogenous nuclear localization signal (NLS), were amplified by PCR and cloned in-frame into the 5′ *Eco*RI and 3′ *Bam*HI sites of the pBTM116 (64) bait plasmid (a gift of Stan Hollenberg, Oregon Health Sciences University, was constructed by Stanley Fields, University of Washington, Seattle, WA and Paul Bartel). The resulting vectors, pLEXA:H-HBD and pLEXA:hge were used in yeast two-hybrid experiments. A third yeast two-hybrid bait vector (pLEXA:HBD) encoding only the HBD of hPR was also constructed by PCR amplification of the HBD as described above and insertion of this fragment into a modified pBTM116 containing the hPR NLS inserted in-frame into the *Pst*I site. A vector encoding the GST fusion protein GST-H-HBD was generated by PCR amplification of the hinge and hormone binding domains of hPR including the entire NLS using primers containing 5′ *Eco*RI and 3′ *Bam*HI sites which were cloned in frame into pGEX 4T1 (Pharmacia, Piscataway, NJ) cut with *Eco*RI and *Bam*HI. The pCMX:mN-CoR construct was a gift from Andreas Hörlein and M. G. Rosenfeld, University of California, San Diego. Full-length L7/SPA was PCR amplified from reverse transcribed HeLa cell cDNA and cloned into the 5′ *Eco*RI and 3′ *Bam*HI sites of the mammalian expression vector pSG5 to create pSG5:L7/SPA. The construct, pLEXA:hN-CoR ID, was made by PCR amplification from the yeast HeLa cell library hN-CoR ID clone of the regions indicated in Fig. 5 and cloned into the 5′ *Eco*RI and 3′ *Bam*HI sites of pBTM116. The hPR1Δ42 mutant was constructed by PCR amplification of a *Hin*dIII-*Bgl*II fragment in the hPR HBD located between amino acids 810 and 891, which was inserted into *Hin*dIII and *Bgl*II-cut hPR1. Wild type hPR and hER expression vectors were obtained from Pierre Chambon (Strasbourg, France), and the hGR expression vector was from John Cidlowski (NIEHS, Research Triangle Park, NC); pCMX-SMRT was a gift from Ron Evans (The Salk Institute, La Jolla, CA). The reporters, PRE_2-$TATA_{tk}$-CAT used for hPR and hGR and ERE_2-$TATA_{tk}$-CAT used for hER, were previously described (65).

Yeast Two-Hybrid System

The plasmid pLEXA:H-HBD was transformed into the yeast two-hybrid reporter strain L40 (64) (MATa his3Δ200 trp1–901 leu2–3, 112 ade2 LYS2::$(lexAop)_4$-HIS3 URA3::$(lexAop)_8$-lacZ GAL4 gal80), a gift from S. Hollenberg, yielding a strain called L40-LEXA:H-HBD. This strain was transfected with a HeLa cell cDNA fusion library cloned into the GAL4 activation domain (AD) vector pGADGH (Clontech, Palo Alto, CA) and plated on appropriate selective media containing 10 μM of the antiprogestin RU486 (Roussel-Uclaf, Romainville, France). Ten million primary transformants were screened for two-hybrid interactions and were detected by growth on histidine drop-out plates and confirmed by β-galactosidase assay. The large-scale library transformation protocol was supplied by Stan Hollenberg and is a modification of published methods (66, 67). Modifications include an overnight growth in liquid media before the histidine selection is applied and the addition of 10 μM RU486 to all growth steps in the transformation protocol.

Yeast β-Galactosidase Assay

Colonies were lifted from original library transformation plates with nitrocellulose filters. Filters were immersed in liquid nitrogen for 15 sec to lyse cells and then placed in petri dishes containing Whatman filters soaked in Buffer ZX (60 mM Na_2HPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.4 mg/ml X-gal, pH 7.0). Reactions were carried out at 30 C for 8 h.

False-Positive Tests

pLEXA:lamin (64) (a gift of Paul Bartel and Stan Fields) was used to test for nonspecific interactions. The positive GAL4 AD library clones were tested for autonomous activation of reporter genes by β-galactosidase assay and by growth on histidine drop-out media in L40. The GST fusion protein GST-H-HBD was expressed and purified according to published methods (68, 69). The hN-CoR ID and L7/SPA proteins were synthesized and labeled *in vitro* (70, 71). Labeled hNCo-R ID and L7/SPA were incubated with purified GST-H-HBD and glutathione Sepharose 4B matrix, pelleted, and extracted, and protein binding was assessed by SDS-PAGE and autoradiography.

Shuttling Positive GAL AD Clones into *E. coli*

A single positive yeast colony was swirled into ice-cold electro-competent HB101 *E. coli* in a 2-cm electroporation cuvette (Bio-Rad Labs, Hercules, CA). Conditions for electroporation were a pulse at 1500 V, 100 W, and 25 milliFarads (mF) followed by a second pulse 30 sec later at 2500 V, 200 W, and 25 mF in a Gene Pulser (Bio-Rad). Bacteria were plated on M9 media lacking leucine.

Cloning of hN-CoR

To obtain full-length hN-CoR, a human HeLa cell 5′ Stretch Plus cDNA Library (Clonetech, Palo Alto, CA) was screened using two probes. The first probe was the original hN-CoR ID isolated by the two-yeast hybrid screen. The second probe was an N-terminal 1540-bp fragment obtained by RT-PCR from HeLa cell total RNA Template, a sense primer based on the mN-CoR sequence (Genbank MMMU35312) beginning at nt 117 (the translation start site), and an antisense primer designed from a human expressed tagged sequence cDNA (Genbank accession N33258, Genome Systems, St. Louis, MO) corresponding to nt 1540 of the mN-CoR. From this screen two N-terminal clones of approximately 2 kb were obtained that contained 350 bp of 5′-untranslated region, and one 1.8-kb C-terminal clone was obtained that started at nt 6890 in the corresponding mN-CoR sequences and contained approximately 1330 bp of 3′-UT. In addition, by RT-PCR of both HeLa and T47D cell total RNA, a 5564-bp fragment was obtained using the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis, IN), and MuLV reverse transcriptase (Perkin Elmer, Branchburg, NJ), using a sense primer beginning at the translation initiation codon of hN-CoR and an antisense primer initiating within the hN-CoR ID obtained from the yeast two-hybrid clone, and corresponding to nt 5564 of mN-CoR. All of the above fragments were sequenced either manually using Sequenase Version 2.0 (Amersham, Cleveland, OH) or with an ABI 377 sequencer (University of Colorado Health Science Center, Cancer Center DNA Sequencing and Analysis Core). The sequence of hN-CoR was assembled using AssemblyLIGN

sequence assembly software (Eastman Kodak Company, Rochester, NY), and compared with mN-CoR using MacVector (Kodak Scientific Imaging, New Haven, CT).

Cell Culture and Transfections

HeLa or COS cells were plated in 100-mm dishes in MEM supplemented with twice charcoal-stripped FCS. Cells were cotransfected by calcium phosphate precipitation (6) with 2 μg reporter plasmid, expression vector (amounts indicated in figure legends), and 3 μg β-galactosidase expression vector pHC110 (Pharmacia-LKB Biotechnology) to normalize for transfection efficiency and carrier DNA for a total of 15 μg/plate. Twenty-four hours after transfection, the cell medium was changed and ligands were added. The following ligand concentrations were used throughout: 100 nM synthetic antagonists RU486 (Roussel Uclaf), ZK98299, ZK112993 (Schering Corp., Berlin, Germany), tamoxifen or ICI164,384 (ICI Pharmaceuticals, Mecclesfield, England) and 10 nM concentrations of the agonists R5020, 17β-estradiol, or dexamethasone. Cells were treated with ligand for 24 h and then harvested. Cell lysates were normalized to β-galactosidase activity, then assayed for chloramphenicol acetyl transferase (CAT) activity by TLC and quantified by phosphorimaging and autoradiography.

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Dedicated to "Grandpa" Bert O'Malley on the happy occasion of his 60th birthday ☺ and to the memory of his first Fellow, William L. McGuire, my teacher and mentor — K.B.H.

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COLOCALIZATION OF POLY(ADP-RIBOSE)POLYMERASE AND THE Ku AUTOANTIGEN/DNA-DEPENDENT PROTEIN KINASE HOLOENZYME ON THE DNA BINDING DOMAIN OF PROGESTERONE RECEPTORS AND REGULATION OF PHOSPHORYLATION IN A DNA-INDEPENDENT MANNER

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ABSTRACT

Progesterone receptors (PR) are phosphorylated, ligand-dependent transcription factors that bind to DNA at specific progesterone response elements (PREs) by means of a DNA binding domain (DBD_{PR}) containing two highly conserved zinc fingers. We have now identified four HeLa cell nuclear proteins that bind to a glutathionine-S-transferase (GST)-human DBD_{PR} fusion, immobilized on a glutathionine-Sepharose 4B matrix. Microsequence and immunoblot analyses identify one of these proteins as the 113 kDa poly (ADP-ribose) polymerase (PARP). The other three DBD_{PR}-binding proteins are subunits of the DNA-dependent protein kinase (DNA-PK) holoenzyme: its DNA-binding regulatory heterodimers consisting of Ku70 and Ku86, and the 460 kDa catalytic subunit, $DNA\text{-}PK_{CS}$. The N-terminal 147 amino acids of the yeast transcription factor GAL4 containing its DBD, also copurify DNA-PK on the affinity matrix. However, neither a DBD_{PR} mutant lacking a structured first zinc finger, nor the wild-type DBD of estrogen receptors interacts with DNA-PK. The ability of each fusion protein to "pull-down" DNA-PK on the affinity resin correlates precisely with the ability of the kinase – while resin bound – to autophosphorylate Ku70, Ku86 and $DNA\text{-}PK_{CS}$; to transphosphorylate the bait DBDs; and to phosphorylate a DNA-PK-specific p53 peptide substrate. Surprisingly, DNase I treatment of bacterial extracts containing the fusion proteins, and of HeLa extracts containing the kinase, does not disrupt the association between DNA-PK and the DBDs, nor abolish its kinase activity. Similarly, addition of DNA, either with or without a PRE, does not further enhance the strong kinase activity seen in the absence of added DNA. Lastly, we find that catalytically active DNA-PK also copurifies with transcriptionally functional, FLAG-tagged, full-length human PR transiently expressed in HeLa cells. This suggests that the human PR/DNA-PK complex assembles *in vivo*. We propose that DNA-PK can assemble, and be brought to the proximity of a promoter through two mechanisms: direct binding to DNA as previously described, and direct binding to the DBDs of transcription factors, as described herein. Cooperativity between the two mechanisms may enhance the binding affinity and kinase activity of DNA-PK at a transcription complex.

INTRODUCTION

Nuclear DNA-dependent protein kinase (DNA-PK) phosphorylates a variety of DNA-binding transcription factors (5,12,29); it is regulated during the cell cycle (56) and plays a crucial role in DNA replication and growth (4,15,37); and it is involved in double-stranded DNA break repair and V(D)J recombination (4,37,62). DNA-PK catalytic subunit (DNA-PK_{CS}) deficiency accounts for the defects in the severe combined immunodeficient (SCID) mouse (4,26,40). The DNA-PK holoenzyme is a trimeric protein complex composed of the DNA-binding heterodimeric regulatory subunits Ku70 and Ku86 and the 460 kDa DNA-PK_{CS} (12,19). Interestingly, Ku86-deficient female mice, while fertile, cannot lactate, suggesting a possible role for DNA-PK in mammary gland development and maturation (18).

As its name implies, DNA-PK is believed to have a unique catalytic requirement for direct DNA contact (5,30). Neither DNA-PK_{CS} nor the Ku heterodimer is active independently, but kinase activity is acquired following assembly of DNA-PK_{CS} onto DNA-bound Ku (12,19). At gene promoter regions Ku heterodimers are thought to first bind single stranded DNA in a single/double strand transition region and then to slide onto double stranded DNA in an energy-independent manner (9,14). Ku binding was thought to be DNA sequence-independent, until several recent studies demonstrated that the affinity of the heterodimers is higher for some specific DNA sequences than for nonspecific DNA (14,16,17,25,38,42). One such Ku-binding DNA element, NRE1, is present in the long terminal repeat of the mouse mammary tumor virus (17,18). It is a sequence-specific DNA binding site for Ku/DNA-PK_{CS}, and indeed, the kinase activity parallels the binding specificity of Ku to DNA. Interestingly, several other heterodimeric transcription factors have been described, including proximal sequence element binding protein PSE1, transferrin receptor element binding protein (TREF), and Nuclear Factor IV, that are closely related to, or identical to Ku (9,27,42,52), and like Ku, PSE1 and TREF proteins have also been shown to bind to specific DNA sequences. Thus, specific DNA binding sites for Ku on gene

promoters may play a more important role in localizing Ku-associated kinase activity than had previously been thought.

Furthermore, two recent studies suggest the possibility that the DNA binding specificity of Ku is dependent not solely on unique DNA binding sites, but also on interactions between the Ku heterodimers and proteins (7,43). The redox factor protein, REF1 (7) appears to be able to bind Ku heterodimers specifically, and at least one other protein, the proto-oncogene $p95^{VAV}$, has been shown to associate with Ku70 (43). Thus, the mechanism by which DNA-PK phosphorylates an extensive array of upsteam and basal transcription factors remains unknown, but may involve protein-DNA as well as direct protein-protein interactions with Ku. The effects of these interactions between Ku heterodimers and other proteins on the catalytic activity of DNA-PK, is also unknown.

Progesterone is a key hormone involved in the development and terminal differentiation of the mammary gland and other female target tissues (8,22, and references therein). Progesterone receptors (PR) are ligand-activated nuclear transcription factors that bind specifically to DNA at progesterone response elements (PREs) (54, and references therein). Binding of the receptors to PREs occurs through a modular DNA binding domain (DBD_{PR}) present in the protein that consists of two fingers, each formed by the coordination of one zinc atom with four cysteine residues. Based upon mutational analyses and the crystal structure of the highly homologous glucocorticoid receptor DBD, residues located in the second zinc finger are postulated to make contact with proteins bound at the activated transcription complex (20,32,47).

DNA-bound PR are *in vivo* and *in vitro* substrates for phosphorylation by several kinases (59, and references therein), at least one of which is dependent on DNA binding of the receptors (53,60). In the current study we demonstrate a novel property of the DNA-PK holoenzyme, namely its ability to interact directly with the DBDs of PR and the yeast transcription factor GAL4. These protein-protein interactions between the Ku heterodimers and these DBDs then recruit $DNA\text{-}PK_{CS}$, whereupon the enzyme acquires catalytic activity in the apparent absence of DNA. We suggest, based on these findings, that DNA-PK can be brought to its substrates on gene promoters

not only by binding to specific DNA sequences as others have shown, but also by binding directly to the DBDs of transcription factors through protein-protein interactions. We propose that cooperativity between the two binding mechanisms may enhance the binding affinity and kinase activity of DNA-PK at a transcription complex. Additionally, the interaction between DNA-PK and PR that we describe, suggests a possible mechanism for the lactational deficiency observed in mice lacking Ku86 (37).

MATERIALS AND METHODS

Plasmid construction

Human PR (hPR) fragments (DBD_{PR}, DBD_{CYS}, AF3, AF3-DBD_{PR}, NT_B-DBD_{PR}, NT_A-DBD_{PR}, DBD_{ER}) were amplified by PCR using pSG5-hPR1 and pSG5-HEGO (gifts from Pierre Chambon, CNRS, Strasbourg, France), pSG5-BUS-DBD (45), and pSG5-$hPR1_{CYS}$ (53) as a template and oligonucleotide primers with either a 5' *Bam*HI or 5' *Bgl*II restriction site and a 3' *Eco*RI site. Amplified fragments were inserted into the corresponding *Bam*HI/*Eco*RI cloning sites of the pGEX-4T-1 or pGEX-2TK bacterial GST gene fusion expression vectors (Pharmacia, Piscataway, NJ) (see Figure 1). Correct fragment insertion was verified by dideoxy sequencing using a 5' pGEX vector primer (Pharmacia). Plasmids containing the Ku70 and Ku86 cDNA inserts (41,61) were kindly provided by Westley Reeves (University of North Carolina, Chapel Hill, North Carolina).

Construction of FLAG tagged hPR_B, and immunoprecipitation assay

To identify proteins interacting with hPR *in vivo*, a construct was created to fuse the hPR to a FLAG epitope at the C-terminal end of the receptor, allowing efficient immunoprecipitaion of hPR with minimal interference at potential protein-protein interacting sites. Full-length hPR_B in the mammalian expression vector pSG5 was cut with *Hind*III and *Bgl*II to remove a fragment containing part of the HBD. This fragment was then recreated by PCR with a forward primer for the *Hind*III site in the HBD and a reverse primer that removed the stop codon, and ligated back into pSG5-hPR1. The fragment encoding the FLAG epitope (GGDYKDDDK) with a C-terminal stop codon was designed with a *Bam*HI site on the 5' end and a *Bgl*II site on the 3' end so that it could be ligated into pSG5-hPR1. The FLAG-tagged hPR_B (f:hPR_B) construct was checked for expression as compared to wild-type hPR_B by immunoblot analysis using the M2 α-FLAG antibody (Kodak, New Haven, CT) after transient transfection of the expression plasmids into COS-1 cells (53). To verify that recombinant f:hPR_B are functional *in vivo*, HeLa cells were

cotransfected with f:hPR$_B$ expression and the PRE$_2$-TATA$_{tk}$-CAT reporter plasmids, and treated with the synthetic progestin R5020. This produced CAT activity equal to that of the wild-type hPR$_B$. For immunoprecipitation, the f:hPR$_B$ plasmid was transiently transfected into HeLa cells, treated with R5020, and whole cell lysates were prepared as described previously (53). Lysates were dialyzed into 0.1 M NaCl, then 100 µl of a 50% slurry of α-FLAG M2 affinity gel was added to 2 mg of protein and rotated at 4°C for 4 hr. The affinity gel was then washed twice with 1 ml of 0.3 M KCl in TEDG, twice with 1 ml of TEDG containing 0.1% NP40 and twice with TEDG. Immunoprecipitated f:hPR$_B$ and associated proteins were boiled in loading buffer, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with α-FLAG M2 monoclonal antibody, antibodies directed against Ku70, Ku86, and DNA-PK$_{CS}$, or an antibody directed against PARP.

GST fusion protein expression

E. coli strain BL21(DE3)pLysS (Novagen,, Madison, WI) was employed for protein expression according to the method described in the GST Gene Fusion System manual provided by Pharmacia Biotech. Overnight cultures were grown from a single colony and diluted 1:10 in 2X YT-G media containing 100 µg/ml ampicillin. Cultures were grown at 37°C until an OD$_{600}$ of 0.6 was reached, at which time isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1.0 mM. Cultures were then grown for an additional 2 hr at 30°C and pelleted for 10 min at 10,000 x g. Bacterial pellets were resuspended in 1 ml of NETGT (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 5% glycerol, 0.1% tween-20) plus protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN) per 10 ml of culture and lysed by mild sonication. The sonicate was cleared by centrifugation for 10 min at 10,000 x g and the supernatant stored at -70°C.

[^{35}S]methionine labeled HeLa cell nuclear extracts

Subconfluent T175 cm^2 flasks of HeLa cells were washed for 30 min at 37°C in methionine-free MEM (Minimal Essential Medium, Sigma Cell Culture, St. Louis, MO), and then incubated for 4 hr at 37°C in methionine-free MEM supplemented with 100 µCi/ml methionine TRANS^{35}S-LABEL (ICN Radiochemicals, Costa Mesa, CA). Cells were collected and homogenized at 4°C in 0.25 ml TEDG (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol) per flask. The cell homogenate was centrifuged for 30 min at 100,000 x g, and the supernatant was stored at -70°C as the cytosol fraction at ~3.0 mg/ml. The protein pellets were resuspended in 0.2 ml of TEDG, 0.4 M NaCl and protease inhibitors (Complete, Boehringer Mannheim) per flask and incubated for 1 hr at 4°C. The resuspended pellet was then centrifuged for 30 min at 100,000 x g and the supernatant was dialyzed overnight in TEDG containing 0.1 M NaCl. The resulting dialysate was stored at -70°C as the nuclear extract, at ~2.5 mg/ml protein. To prepare unlabeled HeLa cell extracts, the same procedure was followed without the addition of [^{35}S]methionine.

GST "pull-down" assay

Bacterial extracts (100µl) containing overexpressed GST-fusion proteins (~10 mg/ml) were rotated with 25 µl of pelleted glutathione-Sepharose 4B affinity matrix (Pharmacia) for 1 hr at 4°C. The beads were repelleted by centrifugation at 100 x g for 3 min, and washed three times with 1 ml NETGT. The immobilized fusion proteins were then rotated with 100 µl of HeLa nuclear extract for 4 hr at 4°C. The beads were again washed three times with 1 ml of NETGT. The bound proteins were eluted by boiling for 1 min in 100 µl protein loading buffer (0.01% bromophenol

blue, 2.5% SDS, 25 mM DTT, 25% glycerol) and resolved by SDS-PAGE on 5-15% gradient or 11% gels. For [^{35}S]methionine-containing samples, the gel was dried onto Whatman paper and visualized by autoradiography.

DNase I treatment of HeLa cell and bacterial extracts

HeLa cell nuclear or bacterial extracts were desalted over a G-25 Sephadex spin column. The column was prepared by spinning 8 ml of a 1:1 slurry of G-25 Sephadex, medium grade (Pharmacia), in 0.1 M Tris, pH 7.5 at 500 x g for 5 min in a 10 ml Econo-column (Bio-Rad, Hercules, CA). One ml was added per 8 ml G-25 slurry and spun at 500 x g for 5 min. Extracts were then diluted with an equal volume of 2x DNase I buffer (300 mM NaCl, 100 mM Tris, pH 7.5, 4 mM $MgCl_2$). DNase I (Sigma, St. Louis, MO) was added to a final concentration of 50 µg/ml and samples were incubated at 4°C or 25°C for 30 min, as described previously (57). Samples were then diluted with an equal volume of 2x NETGT and incubated with the fusion protein bound to the Sepharose 4B affinity matrix, as described above.

Protein microsequencing

GST "pull-down" assays were performed as described above using the GST-DBD_{PR} fusion protein as bait. Proteins were resolved on a 5-15% gradient polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA) in CAPS-methanol buffer (10 mM CAPS, 10% MeOH, pH 11.0). The membrane was stained with Coomassie Blue and the appropriate bands were excised for microsequence analysis. The p110 protein band was N-terminally blocked, which necessitated digestion of the purified protein with lysine endopeptidase. This was accomplished by excising the p110 band directly from the gel, eluting it from the gel slice, and performing the digest in solution. Microsequencing and protease enyme digestions were performed by the NYS Center for Advanced Technology, Cornell University (Ithaca, NY), and the Molecular Resource Center, National Jewish Hospital (Denver, CO).

Immunoblotting

GST "pull-down" assays were performed as described above. Proteins were then transferred to nitrocellulose using a wet transfer apparatus (BioRad) in CAPS-methanol buffer for 5 hr at 0.7 amps in a 4°C cold room. The membrane was blocked with 5% nonfat milk in phosphate buffered saline and 0.1% tween for 1 hr. The mouse monoclonal antibodies N3H10 and N9Cl against Ku, and 18-2, 25-4, and 42-27 against DNA-PK_{cs} were kindly provided by Dr. Richard Burgess (McArdle Labs, Madison, WI), and Dr. Thomas Shenk (Princeton University, Princeton, NJ), respectively. They were used at a concentration of 1 μg/ml as previously described (5,27). A rabbit polyclonal antibody against PARP was purchased from Boehringer-Mannheim. Bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) as previously described (44).

Kinase assay

GST "pull-down" assays were performed as described above to purify the Ku/DNA-PK complex. Aliquots (30 μl) of the glutathione Sepharose 4B matrix containing bound fusion proteins and associated proteins from HeLa cell nuclear extracts were transferred to siliconized 0.65 ml tubes and resuspended in 50 μl of phosphorylation buffer (20 mM Tris pH 7.5, 100 mM KCl, 12 mM $MgCl_2$, 1 mM DTT). Samples were incubated in the absence of DNA, and in the presence of a final concentration of 0.5 μg/ml of an ~250 bp DNA fragment containing two tandem PREs linked upstream of a truncated thymidine kinase (*tk*) promoter sequence, or an ~200 bp DNA fragment containing only the truncated *tk* promoter sequence (45). Three μl of [γ-^{32}P]ATP (12 Ci/mmol) were added to give a final concentration of 5 mM. The reaction mixture was incubated at 30°C for 30 min. The resin was washed once with 0.5 ml NETGT, the proteins were eluted with protein loading dye, and separated by SDS-PAGE. The gel was dried onto Whatman paper and visualized by autoradiography.

Filter binding assay for phosphorylated synthetic peptide

Conditions for the kinase assay were identical to those described above except that 17 μg of a DNA-PK-specific synthetic peptide substrate was added to the incubation mixture. The fusion protein/DNA-PK complex was incubated with the peptide-containing kinase mixture for 10 min at 30°C, after which matrix bound proteins were rapidly pelleted and ^{32}P-labeled peptide separated from free [γ-^{32}P]ATP by a modification of a filter binding assay described previously (29). Briefly, 15 μl aliquots of the supernatant were added to microfuge tubes containing 15 μl of 30% acetic acid to stop the kinase reaction. Quenched kinase reactions were pipetted onto P-81 Whatman 2.5 cm paper circles placed in a Millipore 1225 Sampling Manifold (Millipore) and washed three times with 3 ml aliquots of 15% acetic acid to remove free [γ-^{32}P]ATP. Washed circles were placed in liquid scintillation fluid and retained radioactivity was measured by liquid scintillation spectroscopy. Less than 1% of the radioactivity retained by the circles was due to phosphorylated proteins that dissociated from the Sepharose 4B matrix. The synthetic peptide employed as substrate was obtained from Promega (Madison, WI), and is identical to E^{11} thru L^{25} from the N-terminus of the tumor suppressor protein, p53 (E^{11}PPLSQEAFADLWK*K*), except that T^{18}A and S^{20}A substitutions were made in order to avoid phosphorylation by non-DNA-PK serine/threonine kinases, and a L^{25}K substitution was made to promote binding to P-81 paper (29). The phosphorylated residue is S^{15} located in the DNA-PK-specific SQ^{16} phosphorylation motif (29).

Gel mobility shift assay

Gel mobility shift assays were performed as described (46) using bacterial extracts containing overexpressed GST fusion proteins. [^{32}P]labeled oligonucleotide probes were a 27 bp perfect palindromic consensus PRE, and a 35 bp estrogen response element (ERE) from the *Xenopus* vitellogenin A2 gene promoter (53).

In vitro translation of Ku

The TNT T7 Quick Coupled Transcription/Translation System kit (Promega) with [^{35}S]L-methionine (ICN) was used to *in vitro* translate and label Ku70 and Ku86 from their respective cDNAs.

RESULTS

A diagram showing the structure of FLAG-tagged full-length human PR B (f:hPR$_B$), and of the GST fusion proteins used in this study is shown in Figure 1. The fusion proteins include several PR chimeras encoding the DBD$_{PR}$ alone or DBD$_{PR}$ fused to all or part of the hPR N-terminus; a DNA binding deficient DBD$_{PR}$ mutant in which the structure of the first zinc finger is destroyed by a single missense mutation ($C^{587}A$) that specifically replaces one of the cysteine residues required for zinc coordination (DBD$_{CYS}$); a PR construct containing the strong AF-3 activation domain but lacking the DBD$_{PR}$; GST linked to the DBD of the estrogen receptor (DBD$_{ER}$); and GST linked to the N-terminal 147 amino acids of GAL4 that includes its DBD (GAL4$^{1-147}$).

The ability of several of the receptor constructs to bind DNA at a progesterone (PRE) or estrogen response element (ERE) was tested using the gel mobility shift assay (Figure 2). Bacterial extracts (0.5 or 2.0 µl) containing overexpressed GST fusion proteins were incubated with [^{32}P]labeled double-stranded oligonucleotides containing either a PRE (left) or an ERE (right), and the DNA/protein complexes were resolved by non-denaturing gel electrophoresis, then dried and autoradiographed. The wild-type DBD$_{PR}$ alone (lanes 6,7) or DBD$_{PR}$ linked to AF3 (lane 1) binds well to the PRE. However neither the zinc finger mutant, DBD$_{CYS}$, nor (as expected) the DBD$_{ER}$ bind to the PRE. The DBD$_{ER}$ does bind to an ERE however (lanes 11,12), to which none of the PR constructs can bind. Note in lanes 8-14, that the doublet bands represent non-specific ERE binding proteins present in bacterial extracts, that do not bind to PRE. This study demonstrates that the receptor fragments used in the studies described below, retain appropriate DNA binding ability and specificity.

The present experiments were initially designed to identify coregulatory proteins involved in the strong *in vivo* transcriptional activation properties of the hPR AF3-DBD construct that we had previously described (45). Use of GST-AF3-DBD$_{PR}$ in pull-down assays of HeLa cell extracts led to the isolation of 5 major proteins ranging in size from 62 to >400 kDa. However,

when control constructs were tested, that contained the AF3 or the DBD_{PR} domain in isolation, it became clear that all 5 proteins were bound specifically to the DBD_{PR} and not to AF3 (Figure 3).

In Figure 3A, the GST-DBD_{PR} fusion protein was purified on a glutathione-Sepharose 4B affinity matrix, then incubated with either a nuclear or a cytosolic HeLa cell extract. Matrix-bound HeLa cell proteins were eluted, resolved on an SDS-polyacrylamide gel, transferred to a PVDF membrane and visualized with Coomassie Blue stain. Panel A shows that 5 major protein bands of approximately 62, 80, 110, 200, and >400 kDa were pulled down from the HeLa nuclear extract (lane 2), but were absent in the cytosol (lane 1). Interestingly, DNase I treatment of HeLa nuclear and bacterial cell extracts, prior to performing the pull-down assay, under conditions shown previously to release chromatin-bound Ku70 from isolated nuclei (57), had no effect on the association of the proteins (panel B, compare lanes 1 and 2), suggesting that DNA was not required for these interactions. Figure 3, panel C, shows the proteins that are pulled down by several GST DBD_{PR} fusion proteins from HeLa nuclear or cytosolic extracts labeled *in vivo* with [^{35}S]methionine. Using GST-DBD_{PR}, three major labeled nuclear proteins of 62, 80 and >400 kDa are purified (lane 2) that are absent in cytosols (lane 1). Neither GST alone (lane 3) nor GST-AF3 (lane 5) bind these nuclear proteins, suggesting again that they are bound to the DBD of hPR, and that AF3, when cloned upstream of the DBD_{PR} (lane 4), does not interfere with their binding. The p110 band is only weakly labeled with [^{35}S]methionine, due to its slow metabolic turnover rate (see below).

Identification of GST-DBD_{PR}-associated proteins by microsequencing and immunoblotting

The p62, p80 and p110 bands were excised from the Coomassie stained membrane and processed for N-terminal microsequence analysis. Protein sequence obtained for the p80 band, RSGNKAAVVLCMDVGFT, was perfectly matched in the protein database using the BLAST algorithm, by the 86 kDa subunit of the Ku autoantigen (61). Proteins from the p62 and p110 bands were N-terminally blocked. However, after lysine endopeptidase digestion of the p110

band, sequence was obtained from four internal peptides, HSIRHPDVE, QVRLSK, ANIRVVSEDFLQDV and THATTHNAYDEVI. These were perfectly matched in the protein database to the 113 kDa poly (ADP-ribose) polymerase (PARP) (6). Its weak [^{35}S]methionine labeling is consistent with a slow turnover over rate *in vivo* (10). The identity of the p200 band is under investigation.

Since Ku86 is known to assemble into a trimeric protein complex that includes Ku70 and the 460 kDa, DNA-PK$_{CS}$, it was reasonable to postulate that the p62 and p400 proteins were the other two components of this complex. This was confirmed by the immunoblots shown in Figure 4. After extensive washing, proteins from HeLa nuclear extracts that associated with the purified GST fusion proteins on the glutathione-Sepharose 4B affinity matrix were eluted, and aliquots of the eluate were resolved on parallel 5-15% gradient gels. The gels were either stained with Coomassie Blue or transferred to nitrocellulose and probed with either a combined mixture of antibodies directed against Ku70 and Ku86 (27), or with an antibody directed against DNA-PK$_{CS}$ (8). When nuclear proteins associating with GST-DBD$_{PR}$ were probed with a mixture of antibodies to Ku70 and Ku86, two strongly labeled immunoreactive bands appeared (lane 3, αKu70+86 panel), that comigrated with the Coomassie Blue stained p62 and p80 bands. Similarly, GST-DBD$_{PR}$-associated proteins probed with an antibody directed against DNA-PK$_{CS}$ resulted in the appearance of a single immunoreactive band of >400 kDa (lane 3, αDNA-PK$_{CS}$ panel), the expected molecular weight of DNA-PK$_{CS}$ (21). In contrast, only low levels of immunoreactive Ku70 and no Ku86 or DNA-PK$_{CS}$ were seen to associate with purified GST alone when it was incubated with a HeLa nuclear extract (lane 1, αKu70+86 and αDNA-PK$_{CS}$ panels), suggesting that the DBD$_{PR}$ domain is required for DNA-PK binding. Again, little or no binding was seen with GST-DBD$_{PR}$ when it was incubated with a HeLa cell cytosolic extract (lane 2, αKu70+86 and αDNA-PK$_{CS}$ panels), consistent with the predominantly nuclear localization of these proteins (9,23). The association between the trimeric DNA-PK complex and GST-DBD$_{PR}$ was effectively abolished by a single

$C^{587}A$ point mutation in the DBD to produce GST-DBD_{CYS} (lane 4, αKu70+86 and αDNA-PK_{CS} panels) (53). This mutation destroys the structural integrity of the first zinc finger, resulting in a DNA binding deficient mutant (see Figure 2), and suggests that the zinc finger of the DBD may be directly involved in protein-protein contacts with DNA-PK. Interestingly, each component of DNA-PK also associates strongly with GST-$GAL4^{1-147}$ (lane 6, αKu70+86 and αDNA-PK_{CS} panels), which is known to stimulate DNA-PK mediated phosphorylation of several different substrates (39). Surprisingly, DNA-PK did not associate with GST-DBD_{ER} (lane 5, αKu70+86 and αDNA-PK_{CS} panels), even though DBD_{ER} shares approximately 50% sequence similarity with DBD_{PR} (28), has the same two-zinc finger structure, and full-length human ER are *in vitro* substrates of DNA-PK (2). It is possible that the 68 amino acid DBD_{ER} fragment expressed as a GST fusion used in the present study does not include sufficient flanking sequences for optimal association with DNA-PK, although, as we show in Figure 2, it is able to bind DNA efficiently. It has previously been shown that a minimal GAL4 DBD^{1-94} fragment, containing only DNA binding and dimerization functions, has a reduced ability to stimulate DNA-PK activity compared to the larger, transcriptionally active $GAL4^{1-147}$ construct (39), demonstrating that flanking sequences can be important for its optimal interaction with, and stimulation of, DNA-PK activity. We also confirmed by immunoblot in Figure 4 (lane 7, αKu70+86 and αDNA-PK_{CS} panels) that GST-AF3 lacking DBD_{PR} is unable to pull down the DNA-PK complex. However, association with DNA-PK was not limited exclusively to the DBD_{PR} fragment, since other DBD_{PR} constructs, including GST-AF3-DBD_{PR}, GST-NTB-DBD_{PR} and GST-NTA-DBD_{PR} (see Figure 1), containing various N-terminal PR flanking sequences retained the ability to associate with DNA-PK (data not shown). These hPR N-terminal constructs, particularly GST-AF3-DBD_{PR} and GST-NTB-DBD_{PR}, are strong constitutive transactivators (45). These data suggest that the DNA-PK heterotrimer remains bound to the DBD of PR even when additional PR flanking sequences are present in transcriptionally active constructs.

Figure 4, also shows an immunoblot probed with an anti-PARP antibody (αPARP panel), which confirms the identity of p110 as PARP (3), and its association in HeLa nuclear extracts with purified GST-DBD_{PR} (lane 2). Analogous to the results obtained with DNA-PK, PARP does not associate with GST-DBD_{CYS} (lane 1) or GST-AF3 (lane 6), but it associates strongly with the transcriptionally active GST-AF3-DBD_{PR} fragment (lane 5), and with GST-$GAL4^{1-147}$ (lane 4). GST-DBD_{ER} also appears to have some ability to bind PARP (lane 3).

Kinase properties of the DBD_{PR}-associated DNA-PK complex

To determine the kinase activity of the DNA-PK copurified with GST-DBD_{PR} we measured the autophosphorylation of Ku, which has previously been shown to be dependent on the activated trimeric kinase complex (30). GST-fusion proteins were affinity purified on the glutathione-Sepharose 4B matrix, the beads were washed, and the DNA-PK complex was copurified from a HeLa nuclear extract. The beads were washed again, and DNA-PK bound to GST-DBD_{PR} on the resin was then incubated with kinase buffer containing [γ-^{32}P]ATP at 30°C for 10 min, followed by additional washes to remove free [γ-^{32}P]ATP. The bound proteins were eluted from the Sepharose beads and resolved on a 5-15% gradient SDS polyacrylamide gel (Figure 5). As seen by Coomassie Blue staining in Figure 5, panel A, GST-$GAL4^{1-147}$ (lane 2) and GST-DBD_{PR} (lane 4) effectively pull down Ku70 and Ku86, and to a lesser extent, DNA-PK_{CS}. The parallel [^{32}P]autoradiogram in panel B, lanes 2 and 4, demonstrates that the presence of DNA-PK is associated with autophosphorylation of both Ku subunits as well as of DNA-PK_{CS}. In contrast, GST-DBD_{CYS} (panel B, lane 3) and GST-DBD_{ER} (panel B, lane 5), with which DNA-PK does not copurify, exhibit little or no kinase activity. Note that a low level of kinase activity copurifies with GST-AF3 (panel B, lane 1, open arrowhead), the identity of which is unknown. Figure 5, panel B shows not only the autophosphorylation of the three DNA-PK subunits, but also that the DNA-PK_{CS} transphosphorylates the GST fusion proteins to which the kinase is bound (closed arrowheads).

To map the site of phosphorylation in the fusion proteins, GST was dissociated from the bait peptides by thrombin cleavage, resolved on an 11% gel (Figure 5, panel C), and the position of the [^{32}P]label was monitored (panel D). Free GST migrates as a 29 kDa band (panel C) and the released bait peptides, which range in size from about 10 to 40 kDa, are marked by arrowheads. AF3 migrates as a triplet (open arrowheads). The [^{32}P]label, if any (panel D), appears to comigrate with the released peptides and not with the GST. Note that incomplete thrombin cleavage and/or proteolysis is apparent with GST-DBD$_{PR}$ (panel D, lane 4) and GST-AF3-DBD$_{PR}$ (lane 2) accounting for the additional [^{32}P]labeled bands. The unknown kinase that copurifies with AF3 (panel B, lane 1) generates a triplet banding pattern at the 21 kDa marker (panel D, lane 1) that is characteristic of the phosphorylated state of this receptor domain (45).

To determine whether the DNA-PK heterotrimer that copurifies with the hPR DBD can phosphorylate an exogenous substrate, we measured phosphorylation of Ser^{15} in the N-terminus of the tumor suppressor protein p53, using the modified peptide EPPLSQEAFADLWKK, which is a specific DNA-PK substrate (29). To this end, the DNA-PK heterotrimer was copurified with the GST fusion proteins indicated, using glutathione-Sepharose 4B affinity matrix (Figure 6). The beads were washed, then incubated with the peptide substrate and [γ-^{32}P]ATP at 30°C for 10 min. Reactions were stopped, and transfer of [^{32}P]phosphate to the peptide was quantified by a phosphocellulose filter binding assay (29). Compared to background phosphorylation of the peptide induced by nonspecific binding of kinase to GST alone, phosphorylation of the peptide incubated with the complex of kinase plus affinity purified GST-DBD$_{PR}$ or GST-GAL4$^{1-147}$ was induced 16- and 24-fold, respectively. Only minimal kinase activity was detected with the GST-DBD$_{CYS}$ mutant, but a 4-fold induction was seen with GST-DBD$_{ER}$. These data indicate that the DNA-PK heterotrimer, purified by means of its ability to interact with the DBD of transcription factors, retains its specificity for an exogenously added substrate. Additionally, because of its high sensitivity, this assay suggests that low levels of DNA-PK can bind to the ER DBD, even though this binding was not detected on Coomassie stained protein gels.

DNA-independent kinase activity of DNA-PK?

The experimental procedures used to copurify the DNA-PK heterotrimer from HeLa cell extracts with GST fusion proteins affinity bound to the Sepharose 4B matrix, involves multiple washing steps of the proteins while they are bound to the resin. Little or no DNA would be present under these conditions, yet, as we have demonstrated, DNA-PK retains its kinase activity and specificity. Several investigators have shown that a crude HeLa cell extract lacks sufficient DNA to support the DNA-dependence of DNA-PK kinase activity, so that exogenous DNA has to be added (6,23). The extensive kinase activity we observed in the apparent absence of DNA, and certainly in the absence of exogenously added DNA, was therefore surprising. As shown in Figure 3B, DNase I treatment of the HeLa extract containing DNA-PK, and of the bacterial extract containing the GST fusion proteins, had no effect on selective copurification of the Ku/DNA-PK_{CS} complex. We therefore asked whether addition of exogenous DNA, specifically an ~250 bp fragment containing *tk* promoter sequence and two tandem palindromic PREs (45), would increase the copurification efficiency of the Ku subunits (Figure 7A). In this study, [^{35}S]methionine labeled Ku70 and Ku86 were prepared by *in vitro* translation of each subunit alone (lanes 1-4 and 9-12), or by cotranslation of both subunits (lanes 5-8). They were copurified with either wild-type GST-DBD_{PR} or the DBD_{CYS} mutant using the glutathione-Sepharose 4B matrix. We observed that coexpression of the Ku subunits enhanced their binding to DBD_{PR} compared to the binding of each alone (compare lanes 1, 5 and 9), but that addition of DNA containing two PREs did not increase the levels of DBD_{PR}-bound Ku (compare lanes 5 and 7). Low levels of the Ku subunits also bound to the mutant DBD, due perhaps to their overexpression in the *in vitro* translation system in this experiment.

To determine whether exogenously added DNA augments the catalytic activity of DNA-PK, the trimeric complex was copurified from a HeLa nuclear extract with either GST-DBD_{PR} (Figure 7B, left) or GST-DBD_{CYS} (Figure 7B, right) affinity bound on a glutathione-Sepharose 4B matrix. The copurified proteins were washed extensively and autophosphorylation of the matrix-

bound proteins was assessed, in the absence of exogenously added DNA (Figure 7B, lane 1), or in the presence either of a DNA fragment containing *tk* promoter sequence (lane 2), or the same fragment with two tandem PREs inserted (lane 3). As shown, no kinase activity copurifies with the mutant DBD_{CYS} (right panel). In contrast, with the wild-type DBD_{PR} (left panel), extensive DNA-PK activity is copurified, which transphosphorylates the GST-DBD fusion protein and autophosphorylates Ku70, Ku86 and DNA-PK_{CS} in the absence of exogenously added DNA. Addition of DNA fragments lacking (lane 2) or containing PREs (lane 3) had no significant effect on this catalytic activity.

In vivo association between hPR and DNA-PK

In an effort to determine whether the hPR/DNA-PK multiprotein complex assembles *in vivo*, we transiently transfected HeLa cells with FLAG epitope-tagged, full-length hPR_B (f:hPR_B). To verify that recombinant f:hPR_B are functional *in vivo*, HeLa cells were cotransfected with the f:hPR_B expression and the PRE_2-$TATA_{tk}$-CAT reporter plasmids, and treated with the synthetic progestin R5020. This produced CAT activity equal to that of the wild-type hPR_B (data not shown). The f:hPR_B transiently transfected into HeLa cells were treated with R5020 for 1 hr and immunopurified using anti-FLAG M2 affinity gel. The immunoprecipitated f:hPR_B and associated proteins were transferred to nitrocellulose and probed with αFLAG M2 monoclonal antibody to monitor the efficiency of the immunoprecipitation of FLAG-tagged hPR. The blot was then stripped and reprobed with a mixture of monoclonal antibodies directed against Ku70, Ku86 and DNA-PK_{CS} (Figure 8, αKu+αDNA-PK panel), and then stripped and reprobed with a polyclonal antibody directed against PARP (αPARP panel). All three proteins of the DNA-PK complex copurified with f:hPR_B (lane 1), as did PARP (lane 3). The amount of DNA-PK subunit immunoreactivity present in the crude HeLa cell extract (lane 2), that copurified with f:hPR_B (lane 1) was greatest for Ku70, followed by Ku86 and DNA-PK_{CS}, suggesting perhaps, the order in which the three proteins assemble on PR, and indicating that this assembly occurs *in vivo*. That

Ku70 is the proximal PR binding protein is also suggested by the fact that it was independently isolated from a HeLa cell cDNA library when hPR were used as bait in a yeast two-hybrid interaction screen (data not shown).

DISCUSSION

In this paper we have identified four proteins that interact with the DBDs of hPR and GAL4. They are the 113 kDa PARP enzyme and the three components of the DNA-PK holoenzyme: its Ku70 and Ku86 regulatory subunits and the 460 kDa catalytic subunit. These interactions were discovered because of the association *in vitro* of HeLa cell nuclear proteins with GST-DBD_{PR} and GST-$GAL4^{1-147}$ fusion proteins, and were confirmed *in vivo* with wild-type hPR.

Poly (ADP-ribose) polymerase.

PARP is a eukaryotic nuclear protein that recognizes and binds to DNA strand breaks and is automodified by synthesis of poly (ADP-ribose) (31 and references therein). The enzyme is induced following DNA damage produced by alkylating agents or ionizing radiation (11). Multiple functions have been proposed for this protein including a role in apoptosis (24); binding to histones with concomitant changes in chromatin structure (1); and a signaling role in response to environmental stress (35). Nevertheless, young PARP-deficient mice appear to be healthy and fertile supporting a minor role, if any, for PARP in these proposed functions (58). However, as they age the PARP-deficient animals acquire severe skin lesions and interestingly, the females become obese (58). Could restriction of obesity to females be associated with a role for progesterone; a hormone that has no known function in males? Wang *et al.* (58) have proposed that the relatively mild phenotype associated with PARP deficiency is due to redundancy with an alternative pathway involved in DNA double-strand break repair, and they implicate DNA-PK as the substitute protein in that bypass pathway.

DNA-Dependent Protein Kinase

It is therefore of considerable interest that three other proteins which colocalize with PARP on DBD_{PR} are the components of the DNA-PK holoenzyme. This multiprotein complex of Ku70, Ku86 and $DNA\text{-}PK_{CS}$ exhibits serine/threonine kinase activity (5,12,29), ATP-dependent DNA

helicase activity (55,56), and is implicated in DNA double-strand break repair and V(D)J recombination (4,37,62), DNA replication (15), RNA polymerase dependent transcription (12,17,23), and growth control (37). In addition to having immune system abnormalities arising from defects in double strand break repair and V(D)J recombination, Ku86 deficient mice are 50% smaller than their wild-type litter mates, and while fertile, the females cannot lactate (37). Could the lactational deficiency of females be related to the association between DNA-PK and progesterone? This hormone is a key intermediate in mammary gland development and maturation, and its withdrawal at the end of pregnancy signals the onset of lactation (8,22). Interestingly, the breast cancer susceptibility genes, BRCA1 and BRCA2, have recently been shown to associate with the DNA double strand break repair protein, RAD51, which is functionally redundant with DNA-PK and PARP (50,51). Moreover, Maldonado *et al.* (33) have reported that immune precipitation of RNA polymerase II from HeLa cell nuclear extract copurifies a complex of DNA replication and repair proteins, including DNA-PK and RAD51.

DNA-PK Binding to DBD

Our studies show that PARP and DNA-PK bind to the DBD of hPR and GAL4. The DBD of both proteins have cysteine residues that coordinate the binding of Zn^{++} ions (20,34). These domains were so named, because the first function to be described for them involved DNA binding. However, solution of the GAL4-DBD/DNA crystal structure reveals an open feature in the center of the complex to which another protein could, in theory, bind coordinately (34). The ER and glucocorticoid receptor DBD crystal structures show that the second or carboxy-terminal zinc finger contains residues that make dimer interface contacts, and that other amino acids in this finger extend away from the DNA (20,32,48). These are postulated to be available for interactions with transcription regulatory proteins. Only one such DBD-binding protein has been described to our knowledge, namely $dTAF_{II}110$ whose interaction with hPR maps, at least in part, to the DBD (49), and whose binding may account for the weak transcriptional activity observed with the isolated DBD.

We now describe the binding of a multiprotein complex to GST fusion proteins expressing the DBDs of GAL4 and hPR. This binding appears to be highly specific. There is no binding to GST alone, or to GST fused to the 164 N-terminal amino acids of hPR_B that contain the AF3 domain. Interestingly, binding to the DBD of hER is weak or absent, despite the fact that, like the hPR DBD, it has a two-zinc finger structure (48). As currently defined (13), the two DBDs differ minimally in that DBD_{PR} has 7 amino acids at the N-terminus and 9 amino acids at the C-terminus that the DBD_{ER} lacks, in addition to the core 69 amino acid domain that is shared by both. In fact, bacterially expressed DBD from the *Xenopus laevis* ER which contains a short flanking acidic region is transcriptionally active, suggesting that these flanking residues could, in theory, influence protein-protein interactions (49,36), although we have shown that they do not influence DNA binding (Figure 2). Similarly, Peterson *et al* (39) have shown that sequences flanking the N-terminus of the minimal GAL4 DNA binding and dimerization domains are responsible for enhanced DNA-PK-mediated phosphorylation of transcription factor substrates.

DNA Binding-Independent Activation of DNA-PK?

Our cumulative data suggest that under conditions in which Ku/DNA-PK_{CS} is copurified with DBD_{PR} or DBD-$GAL4^{1-147}$, its kinase activity does not require DNA: (i) DNAse I treatment does not abolish the interaction between the DBDs of the transcription factors and the holoenzyme (Figure 3B). (ii) Addition of DNA does not enhance the protein-protein interaction between the two reactants (Figure 7A). (iii) Addition of DNA with or without specific PRE sequences does not alter the strong kinase activity observed when DNA-PK_{CS} copurifies with DBD_{PR} (Figure 7B). (iv) However, under the identical glutathione-Sepharose 4B matrix "pull-down" conditions, little or no Ku/DNA-PK_{CS} copurifies from a HeLa nuclear extract using GST alone or GST-DBD_{ER} (Figure 5A and B). The latter is particularly noteworthy since the DBD_{ER} binds well to DNA (Figure 2). This control indicates that spurious short DNA fragments that presumably bind with lower affinity to the DBDs of the transcription factors, cannot explain the copurification of Ku/DNA-PK_{CS} that occurs with hPR or GAL4, since the ER DBD would be expected to bind similar DNA fragments.

(v) The Ku/DNA-PK_{cs} complex used in our experiments has been extensively purified (see Figure 3, for example). While it is bound to the Sepharose 4B matrix, most other components of the HeLa cell nuclear extract have been eliminated by extensive washing. Thus, the content of protein and DNA in the solid-phase kinase assay is necessarily lower than that which would be found in the crude extract. Several investigators, using different experimental protocols, have shown that even a crude HeLa cell nuclear extract lacks sufficient endogenous DNA contaminants to support DNA-PK catalytic activity, so that excess exogenous linear DNA must be added (23,60). The fact that under the highly purified conditions used in the present kinase assays, the activity is not augmented by added DNA, suggests that contamination with residual endogenous DNA from the HeLa extracts cannot account for the activity we observe. It is clear that we cannot entirely rule out the possibility that contaminating DNA is responsible for the kinase activity we describe. Nevertheless, given the evidence outlined above suggesting that DNA is not involved, we propose that DNA-PK can be recruited to a transcription complex by an alternative mechanism than that commonly described. We suggest that in addition to its dependence on binding to double-stranded DNA (5,30) and even to specific sequences therein (14,17,25,38,42), DNA-PK can also be brought to the proximity of its substrates by binding directly to transcription factors. Indeed, Maldonado *et al.* (33) have shown that immune precipitation of RNA polymerase II copurifies several DNA replication and repair proteins, including DNA-PK and Rad51, and that the integrity of this complex is unaffected by treatment with DNase I, micrococcal nuclease or ethidium bromide. Finally, Peterson *et al.* (39) have shown that even in the presence of a specific DNA binding fragment, the basal kinase activity of purified DNA-PK_{cs} is low, but can be increased 20-fold by the presence of $GAL4^{1-147}$. While they did not assess possible interactions between $GAL4^{1-147}$ and the DNA-PK holoenzyme, their observations of a role for transcription factors in stimulating DNA-PK activity could be extended by our data that such interactions occur directly, without DNA binding. This model also suggests the possibility that Ku/DNA-PK_{cs} binding to both DNA and to transcription factors cooperatively enhances its binding affinity at a promoter, and enhances its catalytic activity for colocalized substrates.

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FIGURE LEGENDS

Fig. 1. Glutathione-S-transferase (GST) and FLAG epitope tagged bait fusion proteins used in "pull-down" assays. The *top bar* illustrates the domain structure of human progesterone receptors containing a FLAG epitope tag fused to the C-terminus (f:hPR$_B$). The respective translation start sites for hPR B and A isoforms are shown by arrows. AF1, AF2 and AF3 are delineated by line segments representing regions of the receptors that have been shown to contain a transcriptional activation function. DBD: DNA binding domain; H: hinge region; HBD: hormone binding domain. Below the top bar are shown the GST fusion proteins employed. GST-DBD$_{CYS}$ fusion protein contains a $C^{587}A$ point mutation in the first zinc finger of the DBD that completely abolishes DNA binding. GST-DBD$_{ER}$ contains the DNA binding domain of the human estrogen receptor (ER) fused to GST, and GST-GAL4$^{1-147}$ contains the DNA binding domain and N-terminal activation function of the yeast transcription factor, GAL4.

Fig. 2. GST fusion proteins containing DBD$_{PR}$ or DBD$_{ER}$ bind their respective hormone response elements in a gel mobility shift assay. Cell extracts were prepared from *E. coli* strain BL21(DE3)pLysS transformed with the designated GST fusion proteins. Bacterial extract (0.5 or 2.0 µl) was added to a reaction mixture containing either a [^{32}P]labeled double-stranded PRE or ERE oligonuculeotide. NS: non-specific bands obtained with the ERE probe. Free DNA: migration of the unbound oligonucleotide shown at the bottom of the gel.

Fig. 3. Five major HeLa cell nuclear proteins bind to the DBD_{PR} and this binding is unaffected by prior treatment with DNase I. Nuclear or cytosolic extracts prepared from HeLa cells were incubated with GST fusion proteins immobilized on a glutathione-Sepharose 4B matrix. (A) immobilized GST-DBD_{PR} was incubated with cytosolic (C) or nuclear (N) HeLa cell extracts. GST-DBD_{PR} and associated proteins were eluted, resolved by SDS-PAGE on a 5-15% gradient gel, transferred to nitrocellulose and stained with Coomassie Blue. The five associated proteins are labeled according to their approximate size. (B) HeLa cell nuclear extract and the GST fusion protein-containing bacterial extract were either untreated (-) or treated with DNase I (50 μg/ml, final concentration) (+) prior to incubation with the glutathione-Sepharose 4B matrix. After incubation, fusion protein-associated proteins were eluted, resolved by SDS-PAGE, and the gels were stained with Coomassie Blue. Shown are GST-DBD_{PR} and the approximate sizes of major protein bands isolated from HeLa cell nuclear extract that associate with the fusion protein. (C) HeLa cell proteins were labeled *in vivo* with [^{35}S]methionine, after which cytosolic (C) and nuclear (N) extracts were prepared and incubated with GST fusion proteins immobilized on glutathione-Sepharose 4B matrix. Associated proteins were eluted, resolved by SDS-PAGE, transferred to nitrocellulose and autoradiographed. Approximate sizes of major [^{35}S]labeled proteins associated with GST-DBD_{PR} and GST-AF3-DBD_{PR} are shown.

Fig. 4. Confirmation by immunoblot analysis that DBD_{PR}-associated proteins are the trimeric DNA-PK subunits and PARP. HeLa cell cytosolic (C) and nuclear (N) extracts were prepared and incubated with the GST fusion proteins shown, immobilized on glutathione-Sepharose 4B matrix, and eluted proteins were resolved by SDS-PAGE and transferred to nitrocellulose as described in Fig. 3. Shown are the Coomassie Blue stain of the GST-fusion and associated proteins, and immunoblots probed with monoclonal antibodies directed against Ku70 and Ku86 (αKu70+86) and DNA-PK_{CS} (αDNA-PK_{CS}); and a polyclonal antibody directed against PARP (α PARP).

Fig.5. The association of DNA-PK with DBDs correlates precisely with autophosphorylation of Ku and DNA-PK_{cs} and transphosphorylation of the DBDs. HeLa cell nuclear extract was incubated with GST fusion proteins immoblized on glutathione-Sepharose 4B matrix. GST fusion and associated proteins bound to the matrix were incubated with a kinase mixture containing [γ-^{32}P]ATP. After extensive washing, [^{32}P]labeled proteins were then eluted from the matrix, resolved on a 5-15% gradient gel as described, and the dried gel was autoradiographed. (A) Coomassie Blue stain of eluted proteins resolved by SDS-PAGE. *Arrows* denote positions of Ku and DNA-PK_{cs}. (B) Autoradiogram of GST fusion and associated proteins phosphorylated after incubation with [γ-^{32}P]ATP. *Arrowheads* in panels (A) and (B) denote positions of the Coomassie stained and [^{32}P]labeled fusion proteins. *Open arrowhead* in panel (B) denotes activity of an unknown kinase bound to AF3. (C) Incubation with the kinase mixture was followed by thrombin (25 U/ml) cleavage prior to elution from the matrix. Eluted proteins were resolved on an 11% gel and stained with Coomassie Blue. *Arrows* denote the GST protein liberated from the cleaved fusion protein (GST), and a 70kDa bacterial protein that copurifies with the fusion protein. (D) Autoradiogram of dried gel described in (C). *Arrowheads* denote the various DBDs and DBD derivative protein fragments liberated by thrombin cleavage that are Coomassie stained in panel (C) and phosphorylated in panel (D). *Open arrowheads* denote the AF3 triplet that is Coomassie stained in panel (C) and phosphorylated in panel (D) by the unknown kinase (see panel B).

Fig. 6. DNA-PK copurifying with DBD_{PR} phosphorylates a modified peptide substrate of the p53 tumor suppressor protein. HeLa cell nuclear extract was incubated with immoblized GST fusion proteins as described. GST fusion and associated proteins bound to the matrix were washed, then incubated with a kinase mixture containing [γ-^{32}P]ATP and 17 μg of a DNA-PK-specific peptide substrate derived from the N-terminus of the p53 tumor suppressor protein. The matrix-bound GST fusion protein/kinase complex was pelleted and the labeled peptide in the supernatant was separated from free [γ-^{32}P]ATP by filtration over P-81 cellulose phosphate circles. The circles were extensively washed and bound radioactivity was quantitated by liquid scintillation spectroscopy. The amount of peptide phosphorylation for each condition (columns) represents the average of two separate experiments with the range of the experimental values shown (bars).

Fig. 7. Addition of DNA during copurification of DNA-PK and DBD_{PR} has no effect upon levels of bound Ku heterodimer or associated kinase activity. (A) GST-DBD_{PR} was immobilized on glutathione-Sepharose 4B matrix and incubated with [^{35}S]labeled Ku70 and Ku86 that were *in vitro* translated separately (lanes 1-4 and 9-12) or cotranslated (lanes 5-8). Where indicated, 0.5 μg/ml of an ~250bp DNA fragment from the thymidine kinase gene promoter (*tk*), with a tandem PRE_2 insert (PRE_2*tk*), was added to *in vitro* translated Ku prior to incubation with the immobilized fusion protein. The matrix bound complex was eluted, resolved by SDS-PAGE and autoradiographed. *Arrows* denote Ku70 and Ku86 bands. Note that the *in vitro* translated Ku migrated as doublets on SDS-PAGE. (B) GST-DBD_{PR} or GST-DBD_{CYS} was immobilized on glutathione-Sepharose 4B matrix, incubated with HeLa cell nuclear extract, washed, and incubated with a [γ-^{32}P]ATP-containing kinase mixture as described. Where indicated, the kinase mixture contained 0.5 μg/ml of an ~200bp *tk* DNA fragment without a tandem PRE_2 (lanes 2 and 5) or the PRE_2*tk* DNA fragment (lanes 3 and 6) described in (A). *Arrows* denote major phosphorylated proteins including autophosphorylated DNA-PK subunits and transphosphorylated GST-DBD_{PR} fusion protein.

Fig. 8. hPR/DNA-PK complex assembles *in vivo*. Nuclear extract was prepared from HeLa cells transiently transfected with f:hPR_B (1μg). Aliquots of the crude HeLa nuclear extract (lanes 2 and 4), and M2 affinity gel purified f:hPR_B and associated proteins from the transfected HeLa nuclear extract (lanes 1,3 and 5), were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies directed against Ku70, Ku86, and DNA-PK_{CS} (lanes 1and 2), against PARP (lanes 3 and 4), and against f:hPR_B (lane 5), see *arrows*.

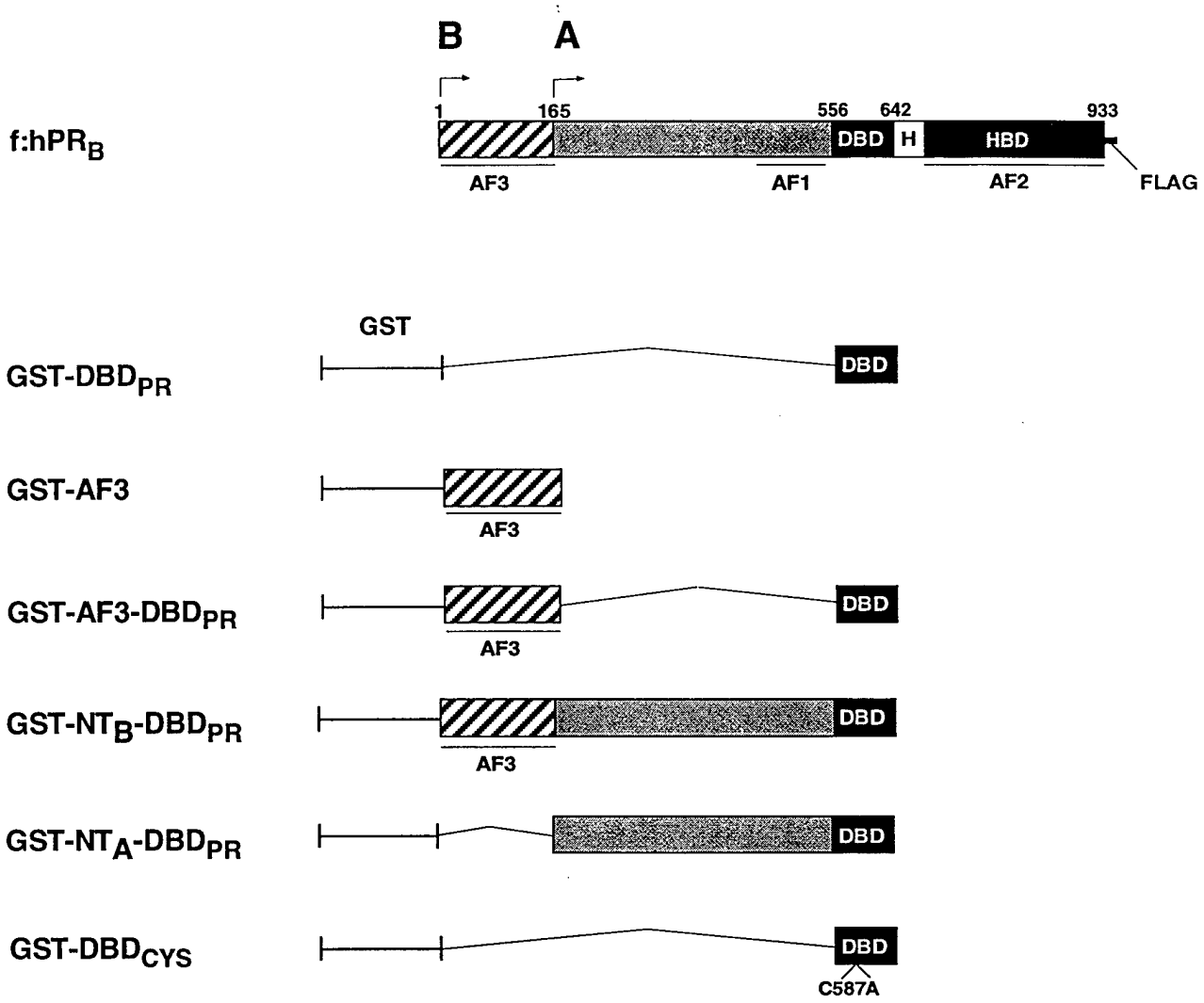

f:hPR$_B$
B
A
1
165
556
642
933
DBD
H
HBD
AF3
AF1
AF2
FLAG
GST
GST-DBD$_{PR}$
GST-AF3
GST-AF3-DBD$_{PR}$
GST-NT$_B$-DBD$_{PR}$
GST-NT$_A$-DBD$_{PR}$
GST-DBD$_{CYS}$
C587A
GST-DBD$_{ER}$
181
250
GST-GAL4$^{1-147}$
94
147

Figure 1

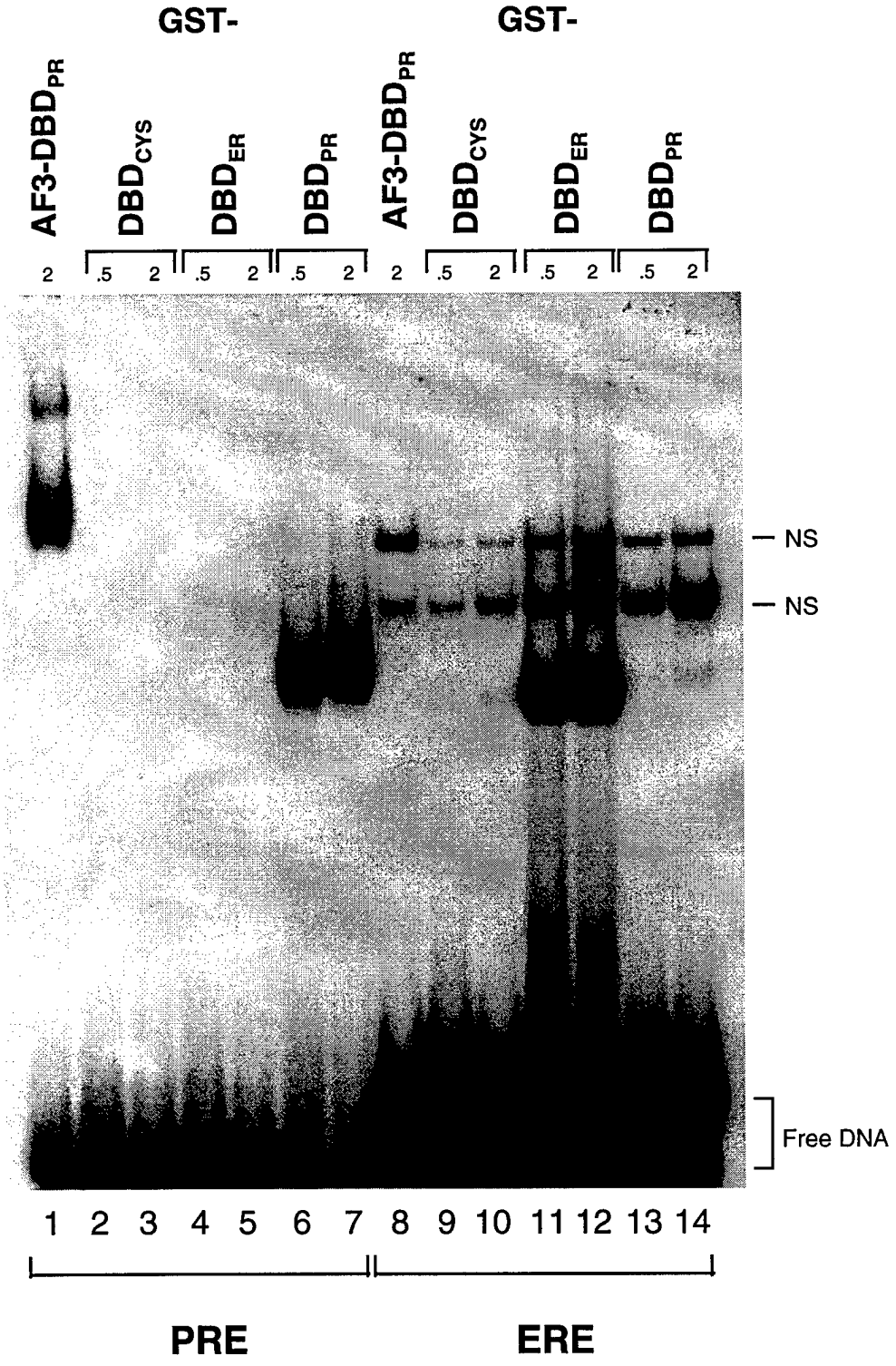

GST-
GST-
AF3-DBD$_{PR}$
DBD$_{CYS}$
DBD$_{ER}$
DBD$_{PR}$
AF3-DBD$_{PR}$
DBD$_{CYS}$
DBD$_{ER}$
DBD$_{PR}$
2
.5 2
.5 2
.5 2
2
.5 2
.5 2
.5 2
NS
NS
Free DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PRE
ERE

Figure 2

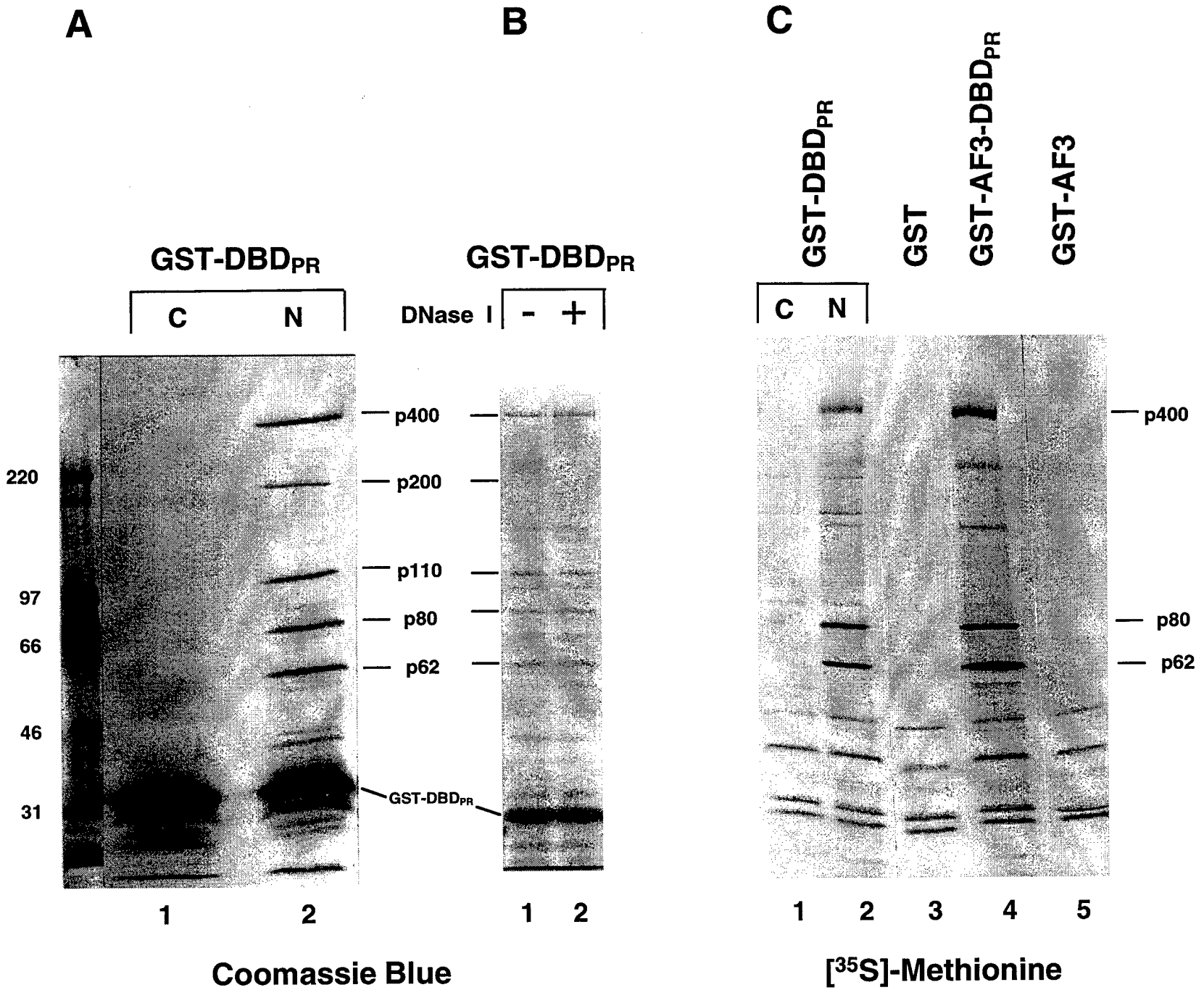

A
B
C
GST-DBDPR
C
N
DNase I
-
+
GST-DBDPR
GST
GST-AF3-DBDPR
GST-AF3
220
97
66
46
31
p400
p200
p110
p80
p62
GST-DBDPR
1
2
1
2
1
2
3
4
5
Coomassie Blue
[35S]-Methionine

Figure 3

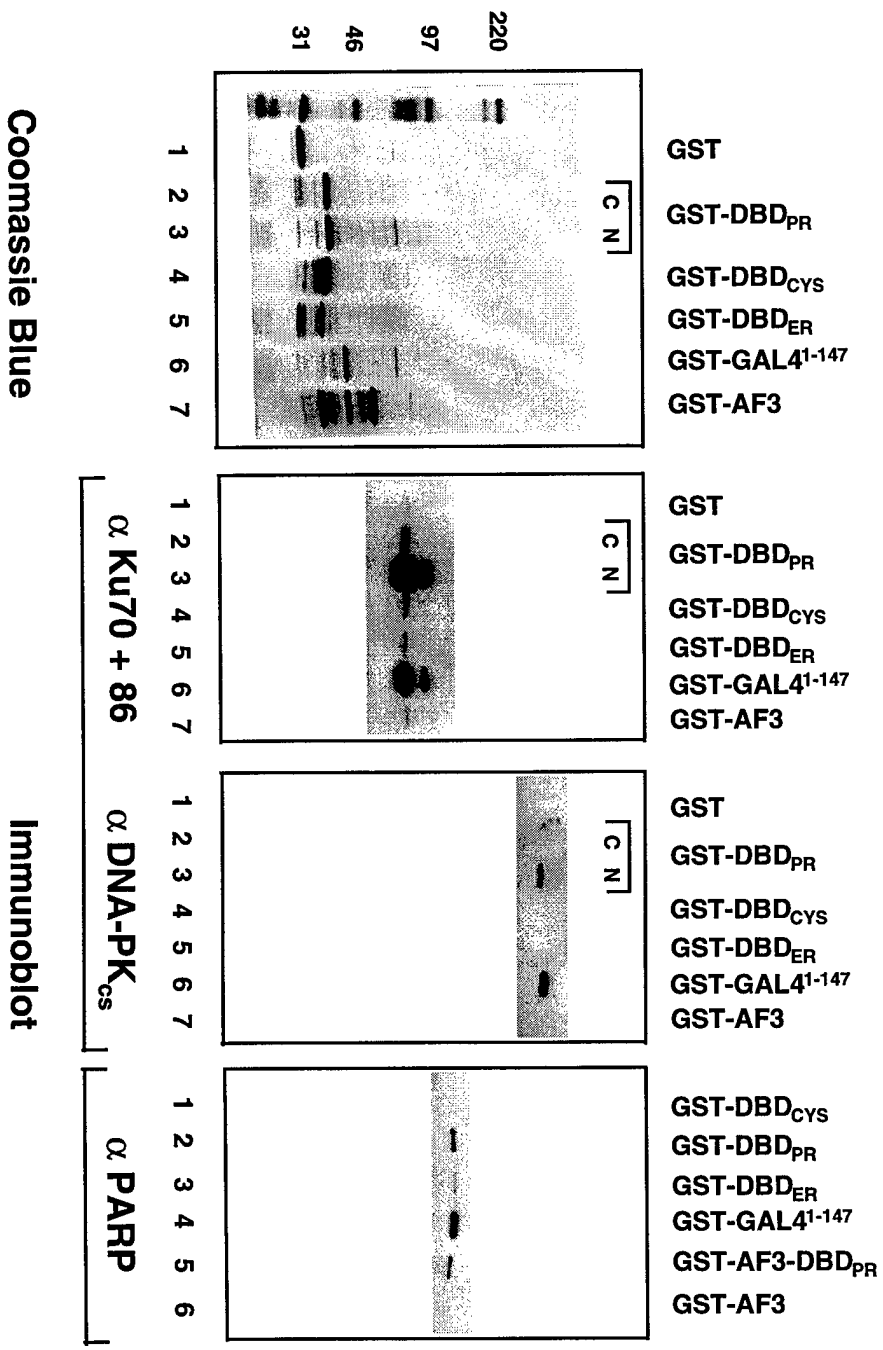

Coomassie Blue
Immunoblot
220
97
46
31
C N
1 2 3 4 5 6 7
GST
GST-DBD$_{PR}$
GST-DBD$_{CYS}$
GST-DBD$_{ER}$
GST-GAL4$^{1-147}$
GST-AF3
α Ku70 + 86
α DNA-PK$_{cs}$
1 2 3 4 5 6 7
GST
GST-DBD$_{PR}$
GST-DBD$_{CYS}$
GST-DBD$_{ER}$
GST-GAL4$^{1-147}$
GST-AF3
α PARP
1 2 3 4 5 6
GST-DBD$_{CYS}$
GST-DBD$_{PR}$
GST-DBD$_{ER}$
GST-GAL4$^{1-147}$
GST-AF3-DBD$_{PR}$
GST-AF3

Figure 4

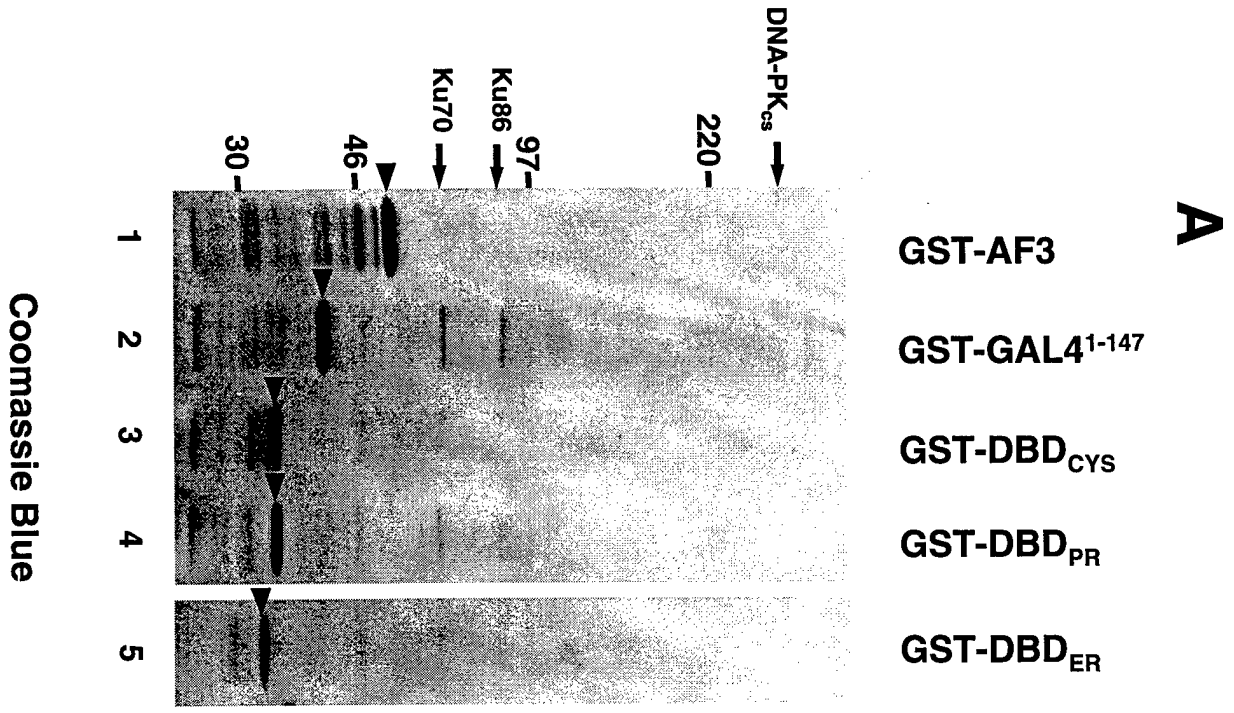


Figure 5A

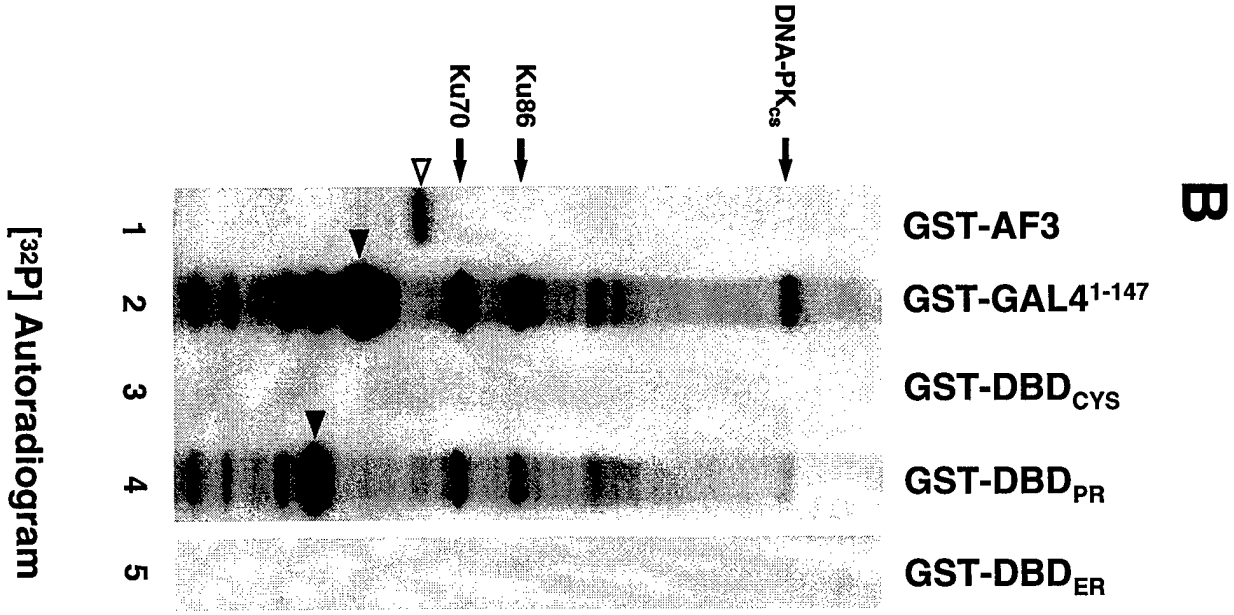


Figure 5B

Figure 5C

Figure 5D

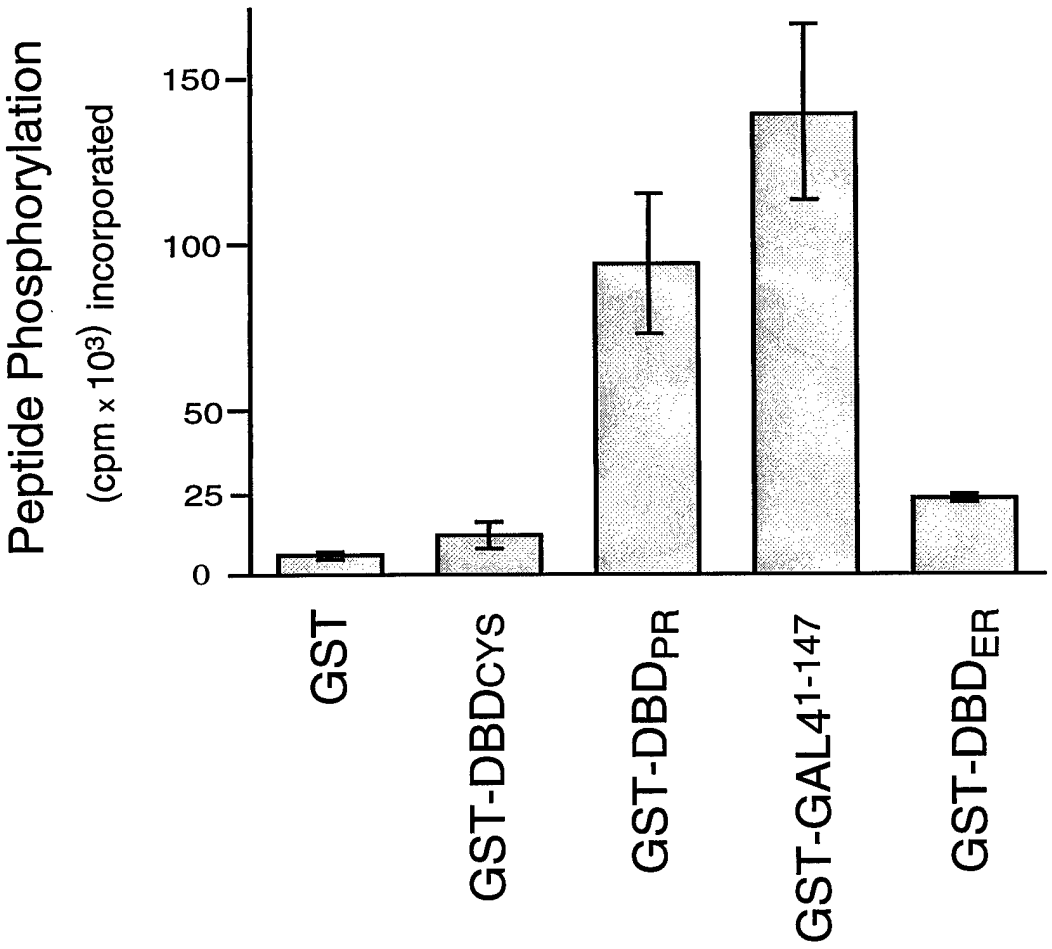
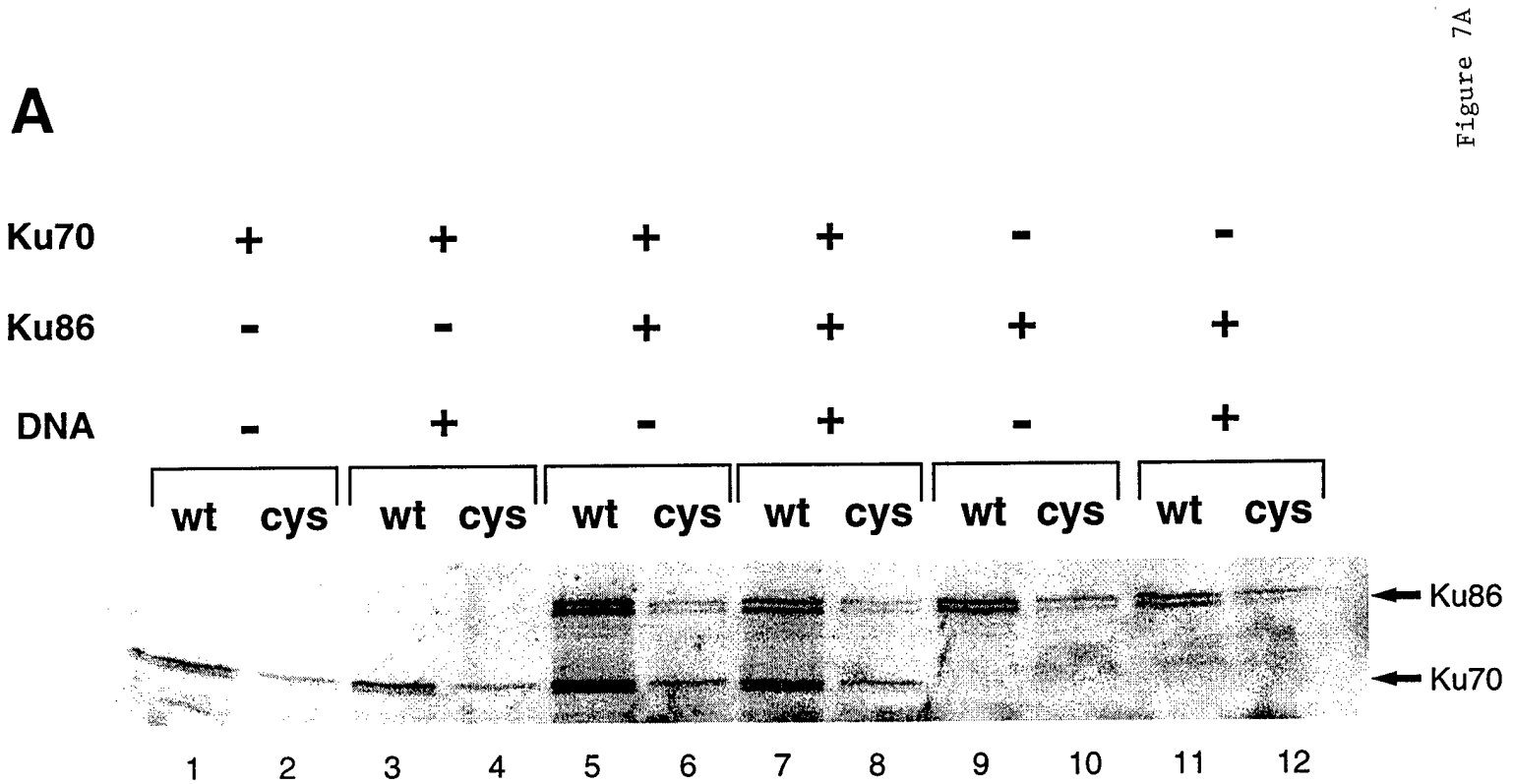

Peptide Phosphorylation
(cpm x 10³) incorporated
150
100
50
25
0
GST
GST-DBDCYS
GST-DBDPR
GST-GAL41-147
GST-DBDER

Figure 6

Figure 7A


A
Ku70 + + + + - -
Ku86 - - + + + +
DNA - + - + - +
wt cys wt cys wt cys wt cys wt cys wt cys
Ku86
Ku70
1 2 3 4 5 6 7 8 9 10 11 12

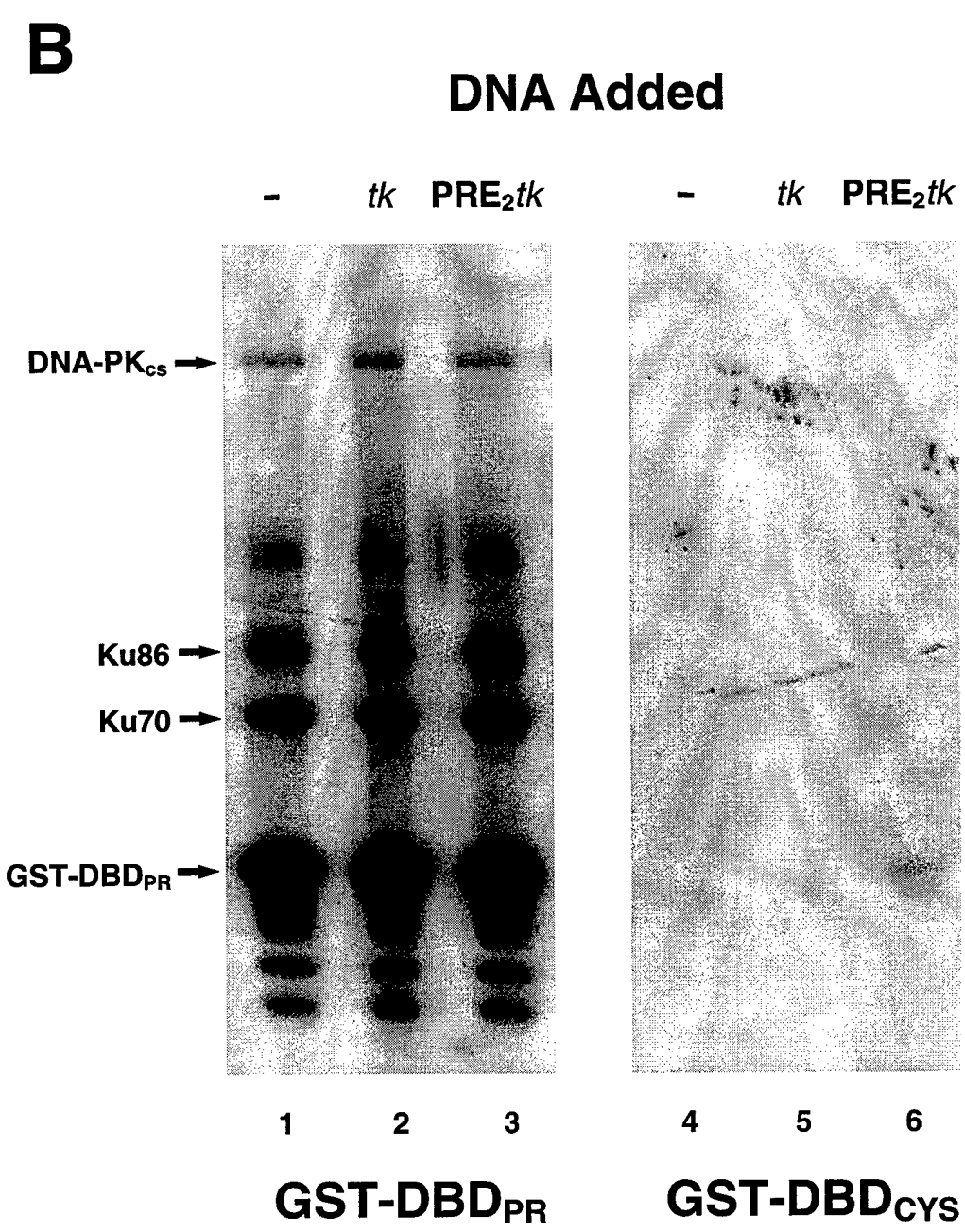

B
DNA Added
- tk PRE2tk - tk PRE2tk
DNA-PKcs
Ku86
Ku70
GST-DBDPR
1 2 3 4 5 6
GST-DBDPR
GST-DBDCYS

Figure 7B

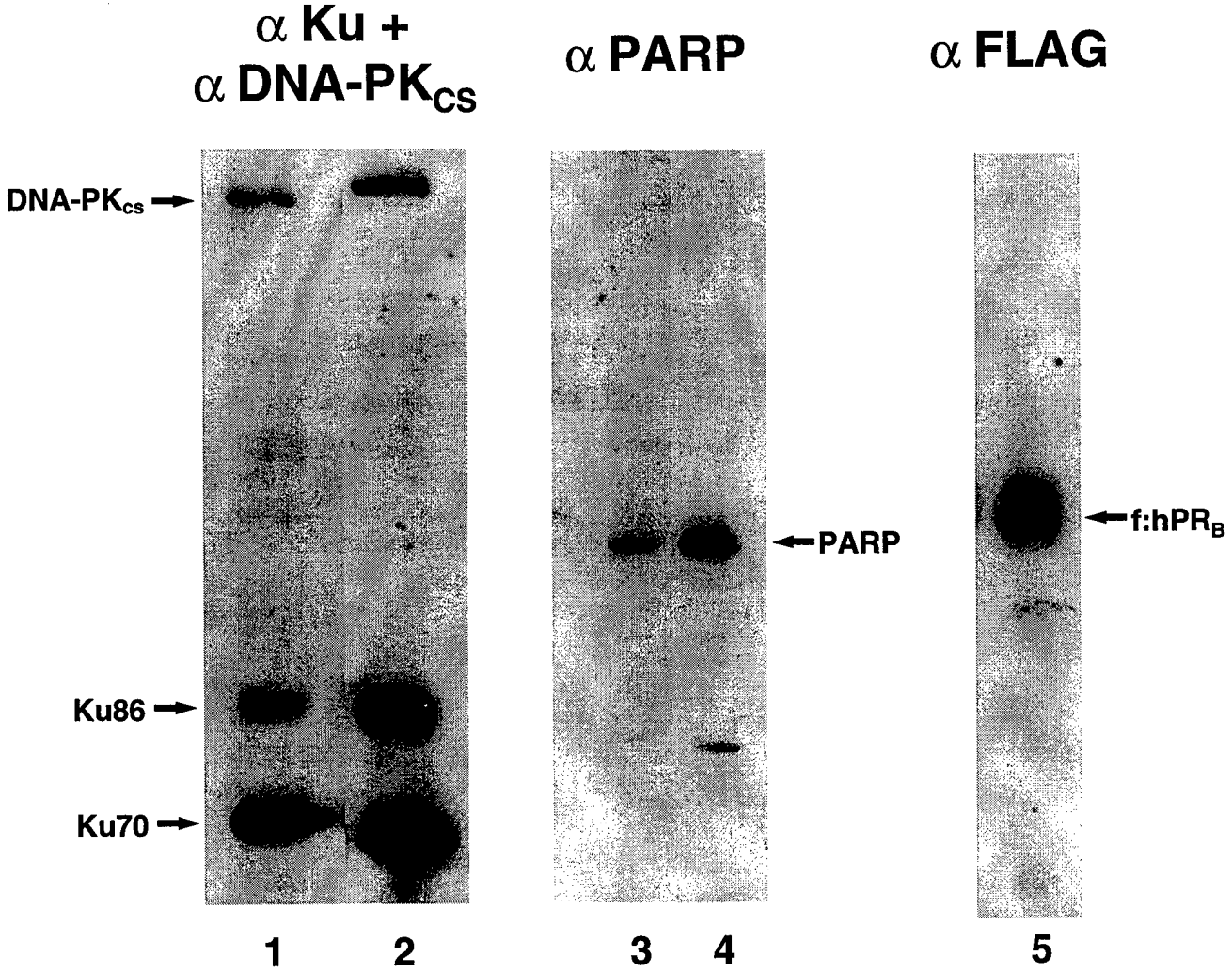

α Ku +
α DNA-PKcs
α PARP
α FLAG
DNA-PKcs
Ku86
Ku70
PARP
f:hPRB
1
2
3
4
5

Figure 8