combination. In addition, our findings raise the possibility that the proteins encoded by the *S. cerevisiae* DNA repair gene *RAD57* (*5*, *18*) and the meiosis-specific gene *DMC1* (*19*), both structurally related to RAD51 protein, may also possess some or all of the RAD51 enzymatic activities described herein.

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9. For overexpression of RAD51 protein in *S. cerevisiae*, the *RAD51* gene from nucleotide position −22 relative to the first ATG translation initiation codon until 180 nucleotides downstream of the TAG termination codon was placed under the control of the yeast alcohol dehydrogenase I (*ADC1*) promoter in the multicopy vector pSCW231 (*20*) to yield plasmid pR51.1. This plasmid was introduced into yeast strain LP2749-9B (*MAT***a**, *his3-Δ1*, *leu2-3*, *leu2-112*, *pep4-3*, *trp1*, *ura3-52*) (*21*).
10. A hybrid polypeptide comprising the COOH-terminal 225 amino acid residues of RAD51 protein fused to the NH_2-terminal 15 residues of the *E. coli* transcriptional terminator ρ was expressed in *E. coli* and purified from inclusion bodies by preparative SDS–polyacrylamide gel electrophoresis (PAGE). Polyclonal antibodies against the ρ-RAD51 fusion protein were raised in rabbits and purified with the use of a Sepharose column containing the ρ-RAD51 antigen. Extract (fraction I) was prepared (*20*) from 100 g of strain LP2749-9B harboring pR51.1. RAD51 and ≈20% of total protein in fraction I were precipitated by ammonium sulfate (0.22 g/ml), redissolved in 150 ml of buffer K [20 mM KH_2PO_4 (pH 7.5), 10% glycerol, 0.5 mM dithiothreitol (DTT), and 0.5 mM EDTA] to give conductivity equivalent to 140 mM KCl (fraction II). This was applied onto Q Sepharose (1.6 cm by 10 cm), which was developed with a 300 ml, 200 to 600 mM KCl gradient. The RAD51 protein peak (fraction III, with conductivity of ≈350 mM KCl) was dialyzed against K + 40 mM KCl and fractionated in Biogel-hydroxyapatite (HTP) (1 cm by 5 cm) with a 75 ml, 0 to 120 mM KH_2PO_4 (pH 7.5) gradient in 10% glycerol, 40 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA. RAD51 protein elutes from HTP at ≈50 mM KH_2PO_4, and the pool of which (fraction IV) was subjected to molecular sizing in Sephacryl S200 (1.6 cm by 35 cm) developed with K + 200 mM KCl. The S200 pool (fraction V) was chromatographed in Mono Q (HR5/5) with the use of a 40 ml, 200 to 500 mM KCl gradient in K. Purified RAD51 (fraction VI; 2.5 mg) elutes from Mono Q at 350 mM KCl and was stored in this buffer (K + 350 mM KCl) at −70°C in 100-μl portions.
11. Purified RAD51 protein was subjected to NH_2-terminal sequencing, but no sequence information could be obtained most probably due to a blocked NH_2-terminus. To circumvent this, three high-performance liquid chromatography (HPLC)–purified peptides derived from a cyanogen bromide digest of RAD51 protein were sequenced instead, and they yielded perfect matches to the following portions of RAD51 protein: (i) residues 23 to 32, (ii) residues 94 to 100, and (iii) residues 284 to 289.
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15. Reaction (final volume, 12.5 μl) contained buffer R [30 mM potassium MES (pH 6.5), bovine serum albumin (100 μg/ml), 1 mM DTT, 2 mM ATP, and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine phosphokinase (1.4 U/ml)], 16 mM $MgCl_2$, 60 ng (180 pmol of nucleotides) of circular viral ssDNA in 1 μl of TE [10 mM tris-HCl (pH 7.5), 0.2 mM EDTA), 50 ng (75 pmol of base pairs) of Pst I–linearized dsDNA in 1 μl of TE, 1 μg of RPA (8.5 pmol) in 1 μl of storage buffer [25 mM tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 1 mM DTT, and 100 mM NaCl], and 2.5 μg of RAD51 protein (58 pmol) in 2.5 μl of storage buffer [20 mM KH_2PO_4 (pH 7.5), 10% glycerol, 350 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA]. To assemble the reaction mixture, we incubated RAD51 protein with viral ssDNA at 36°C for 10 min in the presence of 6 mM $MgCl_2$, followed by the addition of RPA and a 3-min incubation at 36°C, and finally linear dsDNA and 10 mM $MgCl_2$ in 1 μl were added and the reaction proceeded at 36°C for varying times. Reaction mixtures were deproteinized by treatment with 0.5% SDS and proteinase K (1 mg/ml) at 36°C for 20 min and subjected to electrophoresis in 0.8% agarose gels in 30 mM tris acetate (pH 7.4), 0.5 mM EDTA. After staining with ethidium bromide, gels were photographed through a red filter with Polaroid type 55 films. Labeling of 3′ end of Xho I–linearized φX DNA was done with the Klenow polymerase and [α-^{32}P]thymidine triphosphate, and 5′ end-labeling of Pst I–linearized φX DNA was done with T4 polynucleotide kinase and [γ-^{32}P]ATP after removal of the preexisting 5′ phosphate with calf intestinal alkaline phosphatase. In experiments that used radiolabeled DNA, gels were treated with ethidium bromide and then dried onto a sheet of Whatman 3MM paper. The dried gels were examined under ultraviolet light and the positions of various ethidium bromide–stained DNA species were marked with Glo-Bug (x-ray marking solution from Bel-Art Products) on the edge of the gels before exposure to x-ray films.
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Interaction of Dbf4, the Cdc7 Protein Kinase Regulatory Subunit, with Yeast Replication Origins in Vivo

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DNA replication in the budding yeast *Saccharomyces cerevisiae* initiates from origins of specific DNA sequences during S phase. A screen based on two- and one-hybrid approaches demonstrates that the product of the *DBF4* gene interacts with yeast replication origins in vivo. The Dbf4 protein interacts with and positively regulates the activity of the Cdc7 protein kinase, which is required for entry into S phase in the yeast mitotic cell cycle. The analysis described here suggests a model in which one function of Dbf4 may be to recruit the Cdc7 protein kinase to initiation complexes.

The autonomously replicating sequence ARS1 is a chromosomal origin of DNA replication in yeast (*1*) composed of three functional domains designated A, B, and C (*2*). Domains A and B of ARS1 constitute an efficient yeast replication origin (*2*, *3*) that does not activate significant levels of transcription when placed upstream from the *lacZ* reporter gene (*4*) (Fig. 1). Proteins that interact with ARS1 either directly by sequence-specific DNA binding or indirectly by interaction with one or more protein components of initiation complexes might activate transcription when fused to the Gal4 transcriptional activation domain (GAD) (Fig. 1A). This possibility has formed the basis of a genetic screen designed to identify gene products that might interact with an intact and functional replication origin.

A yeast strain containing an ARS1-*lacZ* reporter construct was tested for expression of β-galactosidase after transformation with a library of yeast complementary DNAs (cDNAs) fused to GAD. Five positive clones that expressed β-galactosidase in an ARS1-dependent manner were identified and contained overlapping sequences from the previously characterized *DBF4* gene (*5*) fused in-frame to GAD. One clone, C2 (Fig. 1B), was further characterized. In subsequent experiments, transcriptional activa-

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tion of *lacZ* by C2 was used as an assay for Dbf4-origin interactions in vivo. Dbf4 interacted with all ARSs tested including ARS1, ARS2, the 2-μm plasmid origin, and ARS307 as well as the ARS transcriptional silencer *HMR E* in an orientation-independent manner (Fig. 1C). These results suggest that interaction of Dbf4 with yeast replication origins is a general phenomenon.

Domains A and B of ARS1 have been subdivided by analysis of linker substitution mutations into four functional elements designated A, B1, B2, and B3 (*3*). Mutations in the A element inactivate ARS1, whereas mutations in any single B element result in a functional, but significantly less efficient, origin. The B3 element is a binding site for the transcription factor–origin binding protein ABF1, although the function of the B1 and B2 elements is currently unknown. Domain A serves as the binding site for the multi-subunit origin recognition complex (ORC) both in vitro and in vivo (*6–8*). Genetic analysis has indicated a role for ORC in both DNA replication and transcriptional silencing (*9*). Genes encoding all six subunits of ORC have been isolated; none correspond to *DBF4* (*8–10*).

Mutations in the individual elements of ARS1 affected Dbf4 recruitment (Fig. 2). Inactivation of the A element by linker substitution mutation abolished interactions between Dbf4 and ARS1 (Fig. 2A). Inactivation of either B1 or B2 by linker substitution resulted in a significant reduction in Dbf4 recruitment in vivo, whereas mutation of the B3 element had no effect (Fig. 2A). Double mutations in B1 and B2, which severely reduce ARS function in vivo, caused further reduction in β-galactosidase activity, suggesting that these elements act additively in Dbf4 recruitment (Fig. 2B). Double mutations in B3 and either B1 or B2 resulted in β-galactosidase levels similar to those from single mutations in B1 or B2. Therefore, domain A is essential for recruitment of Dbf4 to ARS1, whereas B1 and B2, but not B3, make significant but nonessential contributions.

Domain A was sufficient for a reduced amount of recruitment of Dbf4 in an orientation-independent manner (Fig. 3A). A series of point mutations in the ARS consensus sequence (ACS), characterized by their effect on ORC binding in vitro and origin function in vivo (*6*), were tested for their ability to recruit Dbf4 to ARS1. The 859 T → A and 863 A → C ACS mutations, which abolish both ORC binding and ARS function, are incapable of recruiting Dbf4 to ARS1, whereas the 860 T → A mutation which results in a significant reduction in ORC binding and ARS function, has a quantitatively similar effect on Dbf4 recruitment (Fig. 3B). Thus, there is a strong correlation among the effects these point mutations have on ORC binding, origin function, and Dbf4 recruitment.

Dbf4 interacts with the Cdc7 protein kinase in vivo, as demonstrated by a two-hybrid assay in which Dbf4 is tethered to a LexA DNA binding domain and Cdc7 is tethered to the GAL4 transcriptional activation domain (*11*). We have used this assay to determine which regions of Dbf4 are required for interaction with Cdc7 and have tested the same Dbf4 deletions for interaction with ARS1 in our one-hybrid assay. Deletions of the NH_2-terminus of Dbf4 eliminate its ability to interact with ARS1 (construct 18) but do not affect its ability to interact with Cdc7 (Fig. 4). Furthermore, deletions from the COOH-terminus of Dbf4 eliminate its ability to interact with Cdc7 (construct 152) but do not affect its ability to interact with ARS1. Thus, the Cdc7 and ARS interaction domains of Dbf4 are not coincident and, therefore, Dbf4 does not interact with initiation complexes through Cdc7. Deletions that eliminate either the Cdc7 or ARS1 interaction can no longer complement a *dbf4* temperature-sensitive mutation, indicating that both the origin- and Cdc7-interacting domains are essential for Dbf4 function in vivo (Fig. 4). Finally, introduction of constructs 152 and 18 together did not complement the *dbf4* temperature-sensitive mutation, suggesting that the Cdc7- and origin-interacting domains must be present on the same molecule (*12*).

Our experiments indicate that Dbf4 interacts with initiation complexes at yeast replication origins in vivo and that interaction requires the binding site for the essential ORC protein. Dbf4 may interact directly with ORC. Alternatively, Dbf4 may in-

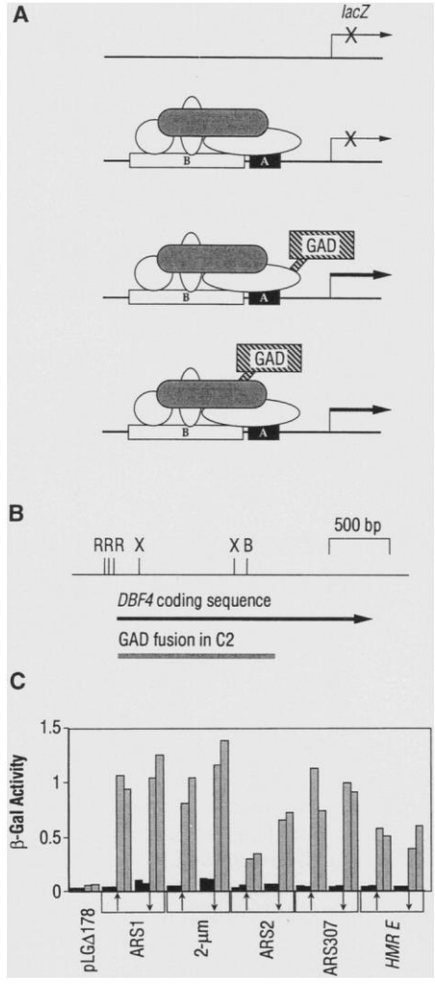

Fig. 1. (**A**) A modified one-hybrid approach (*8*) for the identification of replication origin–interacting gene products. Gene products that interact with yeast origins either directly (shown in white) or indirectly (shown in gray) are expected to activate transcription from *lacZ* when fused to the transcriptional activation domain of GAL4 (GAD). Colonies of cells harboring such fusions are then expected to turn blue in X-gal assays. (**B**) Region of the *DBF4* gene contained in the positive clone C2. One of the five positive clones isolated (C2) contained sequences corresponding to amino acids 4 to 416 of the *DBF4* gene fused, in frame, to GAD. The restriction sites in this map are as follows: R (Eco RI), X (Xho I), and B (Bcl I). (**C**) Interaction of Dbf4 with several yeast replication origins. Levels of β-galactosidase activity in cells from two independent transformants containing C2 (gray bars) or vector alone (black bars) with the indicated ARS reporter constructs in both orientations (arrows) are shown (*22*).

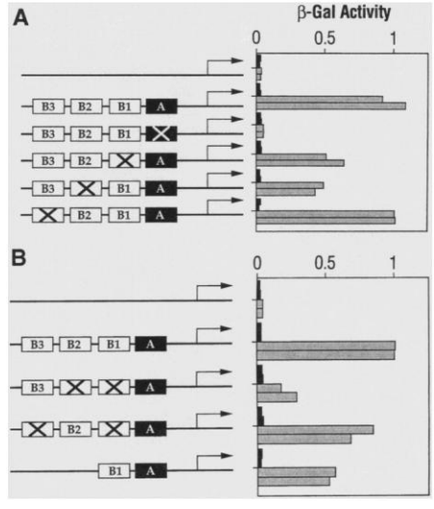

Fig. 2. Domain B (B1, B2, B3) is important and domain A (A) is essential for Dbf4 recruitment to ARS1. (**A**) The effect of mutations in single elements (X) on Dbf4 recruitment to ARS1. (**B**) The effect of mutations in two elements on Dbf4 recruitment to ARS1 (*23*). Reporters containing the indicated ARS1 mutations were tested for their ability to recruit GAD-Dbf4 (C2; gray bars) or GAD alone (black bars) in quantitative β-galactosidase assays (*22*).

teract with an unidentified protein that, in turn, interacts with ORC. In addition to requiring the A element, Dbf4 interaction is also stimulated by the presence of the B1 and B2 elements.

The role of Dbf4 at replication origins is presently unclear. However, given that Dbf4 interacts with and activates the Cdc7 protein kinase (*5*, *11*), we suggest that Dbf4 may function by recruiting active Cdc7 to initiation complexes. Previous biochemical experiments have suggested that a Cdc7-dependent protein kinase, which may be Cdc7 itself, co-purifies with large replication complexes in vitro (*13*). Cdc7-GAD, which can interact with Dbf4 in a two-hybrid assay (Fig. 4), does not activate transcription from an ARS1-*lacZ* construct (*12*). However, Cdc7-GAD does not permit *cdc7* mutant strains to grow at the nonpermissive temperature, suggesting that the molecule is not fully functional. Perhaps the bulky and highly charged transcriptional activation domain prevents Cdc7 from entering initiation complexes. Nonetheless, future experiments are required to determine whether Cdc7 is a component of initiation complexes.

Mutant *cdc7* genes restore silencer activity to certain defective mutant silencers, and overexpression of the wild-type Cdc7 protein causes transcriptional derepression from a wild-type silencer (*14*). As Dbf4 interacts with the *HMR E* silencer in vivo (Fig. 1), this effect may be exerted directly at the level of silencer binding proteins such as ORC, which has been shown to have a role in transcriptional silencing (*9*).

Finally, the use of reporters containing an intact, multi-component, functional sequence (an origin of DNA replication) extends previous two-hybrid and one-hybrid approaches (*8*, *15*) in which binding sites for single proteins were used. The approach outlined here may prove useful in identifying factors that interact with other complex functional sequences such as centromeres, telomeres, and transcriptional silencers.

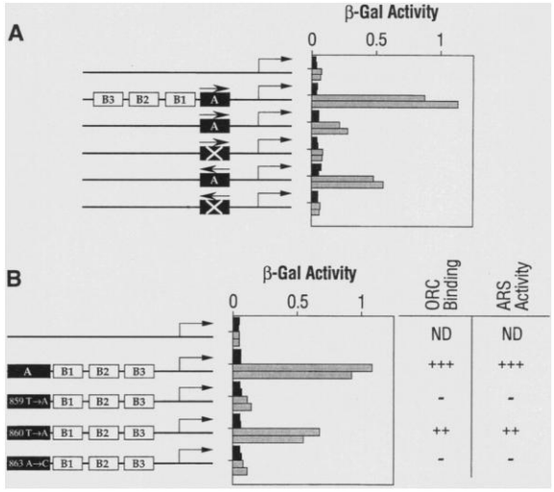

Fig. 3. Domain A is necessary and sufficient for Dbf4 recruitment. (**A**) Reporters containing just domain A from ARS1 in both orientations recruited GAD-Dbf4 (gray bars) to significant levels while mutant domain A (865 to 872 linker substitution) did not, relative to GAD alone (black bars). (**B**) The effect of point mutations in the ACS on Dbf4 recruitment (*24*) correlates with both ARS function and ORC binding, taken from (*6*).

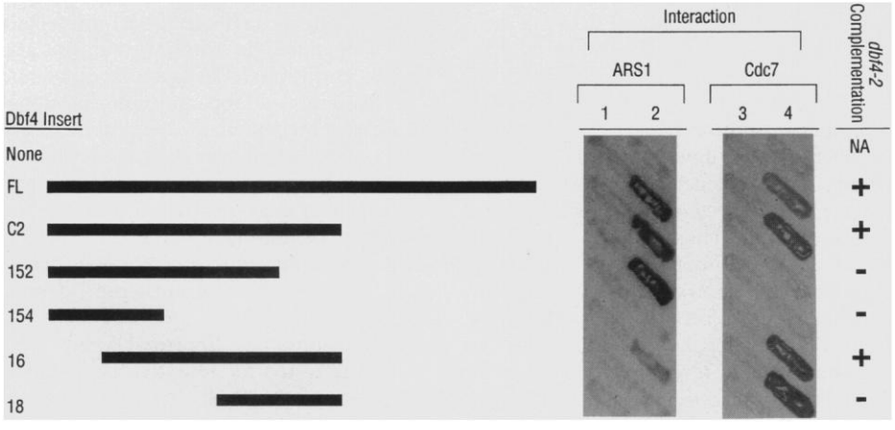

Fig. 4. Dbf4 interacts with origins and with Cdc7 through different domains that are each essential for Dbf4 function. Full-length and various deletions of Dbf4 were tested for their ability to interact with either ARS1 or Cdc7 in a qualitative β-galactosidase assay. For ARS1 interactions, Dbf4 derivatives were fused to GAD and assayed with either pLGΔ178 (column 1) or pLG-ARS1+ (column 2). For the Cdc7 interaction, Dbf4 derivatives were fused to the LexA DNA binding domain in pBTM116 and assayed with either pGAD2F (column 3) or pGAD2F in which full-length Cdc7 was fused to GAD (column 4). Cdc7 interaction experiments had to be done in this way because Cdc7 itself activates transcription when fused to LexA (*11*). The negative control (top row) in both cases is vector without any Dbf4 insert (pGAD2F for ARS1 interaction and pBTM116 for Cdc7 interaction). The LexA and GAD-Dbf4 fusions were all tested for their ability to complement the temperature-sensitive *dbf4-2* mutation (*L202-1A: MAT***a** ura3 leu2 trp1 dbf4-2) at 37°C (*25*).

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22. The strain W303-1a (*MAT***a** ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3,112 can1-100) harboring the plasmid pLG-ARS1+ [domains A and B of ARS1 (*2, 3, 16*) were blunt-ended and cloned into the blunt-ended Xho I site of pLGΔ178 (*17*) in the orientation indicated in Fig. 1A] was transformed with a yeast cDNA library in the vector pACT (*18*). Approximately 10^6 transformants were plated directly onto Hybond N (Amersham) filters on selective medium (yeast ni-

trogen base containing adenine, histidine, tryptophan, and 2% glucose). After approximately 24 hours, filters were transferred to selective plates containing 2% galactose as carbon source and grown for another 20 to 24 hours. X-gal assays were performed directly on these filters (*19*). Positive (blue) colonies were picked from the filter, streaked to isolate single colonies, and reassayed essentially as described above. Blue colonies were plated on selective plates containing uracil and 5-fluoroorotic acid (1 mg/ml) to select against the reporter plasmid. *ura*$^-$ strains were mated to W303-1b (*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*) harboring either pLGΔ178 or pLG-ARS1+. Five clones expressed β-galactosidase with pLG-ARS1+ but not with pLGΔ178. The library plasmids in these strains were rescued into *Escherichia coli* and, upon reintroduction into fresh reporter-containing yeast, retained their original characteristics. The indicated yeast ARSs were first subcloned into pUC plasmids, excised with Eco RI and Hind III, blunt-ended, and cloned into the unique Xho I site of pLGΔ178. The cloning of ARS1 (derived from pARS1.4.1), ARS2, ARS307 (previously called C2G1), and the 2-μm origin as well as the 138–base pair (bp) Dra I–Alu I *HMR E* fragment into pUC plasmids have been previously described (*16*, *20*). All of these inserts are between 100 and 300 bp except for ARS2, which is 627 bp. Quantitative assays for β-galactosidase were performed, and data were normalized to the cell number as described in (*21*). In all experiments, amounts of β-galactosidase were normalized to the amount produced with the wild-type ARS1 reporter.

23. Mutations (indicated as an X) in the individual elements of ARS1 corresponding to linker substitutions in A (865 to 872), B1 (835 to 842), and B2 (798 to 805) and a double point mutation in B3 (756, 758) (*3*) were cloned into pLGΔ178 in the indicated orientation as described in the legend to Fig. 2. To construct the reporter containing elements A and B1 alone (corresponding essentially to a B2, B3 double mutation), an Eco RI–Pst I fragment from pARS1-WTB(*3*) was blunt-ended and cloned into the blunt-ended Xho I site of pLGΔ178. Quantitative β-galactosidase assays were performed as described above (*22*).
24. Domain A from either wild-type or mutated (865 to 872 linker substitution) ARS1 (*3*) was excised as an Eco RI–Bgl II fragment and cloned into pLGΔ178 as described in the legend to Fig. 1. Arrows indicate the orientation of the ACS. The arrow pointing right represents the ACS in the same orientation as it is in the intact ARS1. In (B), the wild-type domain A was reconstructed by the cloning of the double-stranded oligonucleotide:

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5′GATCTAAACATAAAATCTGTAG 3′
    |||||||||||||||||||
3′ ATTTGTATTTTAGACATCTTAA 5′
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between the Eco RI and Bgl II sites of pARS-WTB (*3*). This procedure removes sequences 5′ to the T-rich strand of the ACS that are not necessary for ARS function (*3*). Point mutations were constructed in the same manner, except that the double-stranded oligonucleotide contained the indicated point mutations. In each case, the wild-type or mutant ARSs were subcloned into pLGΔ178 and quantitative β-galactosidase assays were performed as described in (*22*). Levels of ARS activity and ORC binding were taken from (*6*).
25. Dbf4 deletions were generated by polymerase chain reaction with the use of oligonucleotides with Bam HI sites for subcloning PCR products into the relevant vectors. The constructs contained the following portions of Dbf4: Full-length (FL), amino acids 1 to 695; C2, amino acids 4 to 416; 152, amino acids 1 to 320; 154, amino acids 1 to 160; 16, amino acids 81 to 416; and 18, amino acids 241 to 416. Because the two interaction assays are quite different, only qualitative X-gal assays are shown. Construct 16 reproducibly activates significant, albeit reduced, levels of β-galactosidase activity in this assay, suggesting a reduction but not elimination of ARS interaction capability. All constructs were tested for their ability to complement a *dbf4-2* mutant at 37°C. In no case was there a significant difference between the lexA and GAD fusions (*12*).
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Convergent Pathways for Steroid Hormone– and Neurotransmitter-Induced Rat Sexual Behavior

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Estrogen and progesterone modulate gene expression in rodents by activation of intracellular receptors in the hypothalamus, which regulate neuronal networks that control female sexual behavior. However, the neurotransmitter dopamine has been shown to activate certain steroid receptors in a ligand-independent manner. A dopamine receptor stimulant and a D_1 receptor agonist, but not a D_2 receptor agonist, mimicked the effects of progesterone in facilitating sexual behavior in female rats. The facilitatory effect of the neurotransmitter was blocked by progesterone receptor antagonists, a D_1 receptor antagonist, or antisense oligonucleotides to the progesterone receptor. The results suggest that in rodents neurotransmitters may regulate in vivo gene expression and behavior by means of cross-talk with steroid receptors in the brain.

Sexual receptivity in the female rat depends on the ovarian steroid hormones estrogen (E) and progesterone (P) (*1*). The effects of E and P on sexual behavior in rodents appear to require their interaction with specific steroid hormone receptors located in the ventromedial nucleus of the hypothalamus (*2*). The receptors are thought to mediate the actions of these hormones by functioning as ligand-dependent nuclear transcription factors that alter the expression of specific genes or gene networks in the hypothalamus (*3*). The time course of induction and decay of sexual behavior correlates with the amount of inducible progesterone receptor (PR) in the ventromedial nucleus region (*4*). The progesterone antagonist RU 38486, administered before P, inhibits lordosis in E-primed rodents in a dose-dependent manner, which indicates that P occupies its classical nuclear receptor (*5*). Thus, certain actions of steroid hormones in the brain apparently result from activation of intracellular receptors that regulate behavioral functions by modulating gene expression (*6*).

Certain steroid receptors, such as avian PR, human estrogen receptor (ER), and vitamin D_3 receptor, can be activated in a ligand-independent manner by the neurotransmitter dopamine in cultured cells (*7*). Dopamine has no detectable affinity for intracellular steroid receptors and may act by altering phosphorylation of either the receptor itself or a specific transcription cofactor. A single amino acid mutation in the COOH-terminal region of either PR or ER eliminates activation by dopamine but not by the authentic cognate ligand. This ligand-independent activation of certain steroid receptors can also be induced by certain growth factors (*8*). To corroborate that dopamine activation of a steroid receptor has physiological relevance in the central nervous system, we used an established P-dependent behavioral model in female rats. Dopaminergic transmission also has effects on this model because hypothalamic administration of dopamine, or the dopamine receptor stimulant apomorphine, exerts a stimulatory effect on sexual behavior in E-primed female rats (*9*). We examined the effects of inhibition of PR gene expression on dopamine-facilitated sexual behavior of female rats by intracerebroventricular (icv) administration of antisense oligonucleotides to the PR.

Ovariectomized Sprague-Dawley rats previously tested for receptive behavior in the presence of sexually active males were stereotaxically implanted with stainless steel cannulae into the third ventricle to facilitate direct icv injection (*10*). When these animals were primed with E subcutaneously and P was administered by icv injection 48 hours later, they exhibited a high frequency of lordosis [scored and represented by the lordosis quotient (LQ)] in the presence of males (Fig. 1A). Apomorphine, a dopamine receptor stimulant, administered by icv injection 48 hours after E-prim-

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