pool; although our results do not rule out the existence of a small reserve pool, our results can be quantitatively described by a simple model involving spatially random recycling into a uniform pool of vesicles.

How could vesicle mixing occur? Vesicles are clearly constrained at rest in discrete clusters, and evidence suggests that, within each cluster, individual vesicles are bound to a filamentous meshwork consisting, at least in part, of the protein synapsin I (*4, 15*). It has been suggested that during activity vesicles detach from synapsin I (*16*), but the observation that fluorescent dye spots do not blur during destaining (*2*) suggests that vesicles are still constrained in some way during activity. If they are actively moved to the presynaptic membrane (for example, by a molecular motor linked to the cytoskeleton), the present results suggest that recycling vesicles must be able to enter this queue at any location. This could happen if, for example, mature vesicles reappeared [for example, by budding from cisternae and by the removal of clathrin coats (*1*)] at random locations within the cluster.

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5. Experiments were performed on acutely dissected frog cutaneus pectoris nerve muscle preparations as described (*2*). Briefly, muscles were stained by exposure to FM1-43 (1 to 2 μM) or RH414 (40 μM) in bathing solution containing 60 mM KCl, 55 mM NaCl, 1.8 mM $CaCl_2$, and 2.4 mM $NaHCO_3$ and then washed in normal Ringer's solution (2 mM KCl, 115 mM NaCl, 1.8 mM $CaCl_2$, and 2.4 mM $NaHCO_3$). For the experiments in Fig. 1, $[K^+]$ was 25 mM during staining, and terminals were exposed to dye for 2 min (partial load) or 5 min (full load). We used curare (3 to 9 μM) to block muscle contractions. Images were obtained with a Leitz Laborlux epifluorescence microscope with a 100-W Hg lamp and 1 to 10% neutral density transmission filters. Carl Zeiss ×40 water immersion and ×63 oil immersion objectives (0.75 and 1.4 numerical aperture, respectively) were used. Optics for FM1-43 included a 430- to 440-nm band-pass excitation filter, a Leitz H3 dichroic mirror, and a 500- to 600-nm band-pass emission filter. Optics for RH414 included a 541- to 551-nm band-pass excitation filter, a Leitz N2 dichroic mirror, and a 600- to 700-nm band-pass emission filter. Optics for FM1-43 in Fig. 1C included a 450- to 550-nm band-pass emission filter. Control observations of terminals stained with only one dye showed that spectral separation of the two dyes was virtually complete under these conditions. Under optimal conditions, RH414 is invisible in the 450- to 550-nm range, but FM1-43 can be detected in the 500- to 600-nm range. Thus, terminals were stained and imaged first with RH414. Only terminals on surface muscle fibers were studied. Images were captured with a Star I camera (3-s exposure; gain 4) (Photometrics Ltd., Tucson, AZ) and were stored and processed with a Personal Iris computer (Silicon Graphics, Mountain View, CA) running software from G. W. Hannaway and Associates (Boulder, CO). Images in any single destaining experiment were processed identically and aligned. We marked spots to be measured on the first image, and average spot intensities were then computed for each image. We used conventional intracellular recording methods to measure EPPs. Overillumination slows dye release, but the safety factor is large enough to ensure that many consecutive images can be obtained without signs of phototoxicity (*2*). In these experiments, illumination times and intensities were well below levels at which such effects are observed.
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Dimerization of a Specific DNA-Binding Protein on the DNA

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Many specific DNA-binding proteins bind to sites with dyad symmetry, and the bound form of the protein is a dimer. For some proteins, dimers form in solution and bind to DNA. LexA repressor of *Escherichia coli* has been used to test an alternative binding model in which two monomers bind sequentially. This model predicts that a repressor monomer should bind with high specificity to an isolated operator half-site. Monomer binding to a half-site was observed. A second monomer bound to an intact operator far more tightly than the first monomer; this cooperativity arose from protein-protein contacts.

THE BINDING SITES FOR MANY SPECIFIC DNA-binding proteins have dyad symmetry (*1, 2*), and x-ray crystallographic studies of several DNA-protein complexes show that one subunit of a dimer contacts each half of the operator (*2, 3*). For many proteins, these complexes form by binding of preformed dimers to their sites. It is not certain, however, that this is true in all cases. Indeed, several such proteins do not detectably dimerize in solution (*2*), or they dimerize weakly as in the case of LexA, a bacterial transcriptional repressor (*4, 5*).

LexA, which represses the SOS regulon (*6*), is similar to the cI repressor of bacteriophage λ, both in the sequence of its COOH-terminal domain and in its general organization. Its NH_2-terminal domain binds DNA (*7, 8*); the COOH-terminal domain provides most of the contacts for dimerization, because the in vitro dissociation constant for dimer formation in solution (K_{dimer}) is about the same for both the COOH-terminal domain and intact LexA (*4, 5*). However, the value of K_{dimer}, ≈50 μM, is so high that the concentration of dimers in in vitro experiments is low. This suggests that the pathway for binding of dimers to DNA (Fig. 1A, pathway I) cannot account for the rapid rate at which LexA

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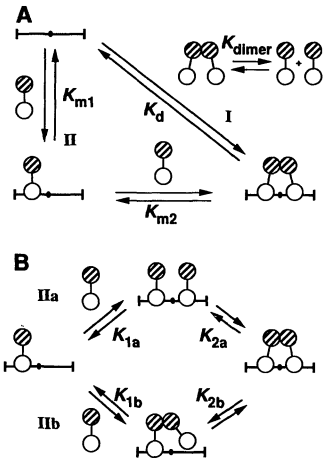

Fig. 1. Pathways for LexA binding to a site with dyad symmetry. (**A**) Alternative pathways for binding. Repressor monomers are depicted as dumbbells. The DNA-binding domain and the domain allowing dimerization contacts are represented by open and shaded circles, respectively. The binding site on the DNA is shown as a line with the two half-sites separated by a filled oval on the dyad axis. In pathway I, repressor dimers form in solution and bind to the operator; in pathway II, two monomers bind sequentially to the operator (*12*). (**B**) Two steps in binding of the second monomer in pathway II. In pathway IIa, the protein-DNA contacts are made first; in pathway IIb the protein-protein contacts are made first. The equilibrium constants shown for the reactions are dissociation constants (*21*).

binds to DNA in vitro, and that a monomer binding pathway (Fig. 1A, pathway II) might be operative (*9*).

Pathway II predicts that a LexA monomer should bind to a half-operator. To test this prediction, we synthesized a half-operator that corresponded to the left half of a strong binding site, the *recA* operator (Fig. 2A). Deoxyribonuclease I (DNase I) footprinting analysis of DNA containing this half-site, and of DNA with the full *recA* operator, was carried out as a function of LexA concentration; we determined the affinity by measuring the LexA concentration that gave half-maximal protection (*10, 11*) (Table 1, legend).

LexA repressor bound to the half-operator (Fig. 2B), but not to the nonoperator sequences that replaced the other half of the operator. This finding is consistent with pathway II. At several salt concentrations, LexA bound ≈1000-fold more tightly to the intact operator than to the half-site (Table 1), indicating (*12*) that the second monomer bound about 10^6-fold more tightly than the first one.

Cooperative binding such as we observed for the second monomer is generally believed to arise from protein-protein interactions between adjacent bound proteins. It is possible, however, that the first bound monomer confers a conformational change on the DNA-binding site that increases the affinity of this site for the second monomer. Consequently, some of the energy of cooperativity might be provided by effects on DNA structure. We took advantage of the domain structure of LexA to test this possibility.

Because the contacts for LexA dimerization in solution reside in the COOH-terminal domain (*5*), a DNA-bound NH_2-terminal domain should not provide a surface for protein-protein interactions. Accordingly, the affinity of the NH_2-terminal domain for the intact operator should be the same as for the half-operator, if its binding had little or no effect on DNA structure. This prediction was fulfilled (Table 1). Thus, we conclude that essentially all the energy for cooperative binding of the second monomer of intact LexA comes from protein-protein interactions, and that these take place in the COOH-terminal domain.

Two lines of evidence strongly suggest that intact LexA bound to the half-site as a monomer. First, sequences replacing the other half of the operator were not protected from DNase I (Fig. 2B) (*13*). Second, the isolated NH_2-terminal domain had the same affinity for the half-site (Table 1), and gave the same footprint (Fig. 2B), as did intact LexA. These findings also suggest that the contacts between the half-site and either form of repressor were identical.

In addition to this evidence favoring pathway II, we argue against pathway I on kinetic grounds. LexA binding to an intact operator was essentially at equilibrium within 20 s (*14*). Pathway I cannot account for this rapid approach to equilibrium because the dimer concentration in solution is so low that dimers cannot bind rapidly enough (*15*). We conclude from all our data that LexA binding largely follows pathway II under our conditions. Studies with the DNA-binding domain of the glucocorticoid receptor suggest that it can dimerize on the DNA (*16, 17*), with the second monomer binding ≈100-fold more tightly than the first (*17*).

Genetic evidence obtained at high concentrations of LexA in vivo has been interpreted to indicate that pathway I operates under physiological conditions (*18*). Negative-dominant mutant forms of LexA with alterations that block DNA binding interfere with the function of wild-type LexA, presumably by forming nonfunctional heterodimers. This has suggested that mutant monomers deplete the cell of wild-type monomers and therefore of wild-type homodimers. However, these experiments do not identify the DNA-binding species but simply indicate that its concentration is reduced. This would be true both of wild-type homodimers (which bind in pathway I) and of wild-type monomers (which bind in pathway II). Although all the equilibria in Fig. 1A must be satisfied, pathway I may be kinetically blocked—the rate constants in both directions may be small—so that the contribution of the pathway would be insignificant.

Table 1. Affinities of LexA for intact (I) and half-operator (HO). Samples were prepared and analyzed as in Fig. 2B, with a range of protein concentrations. Footprinting gels were analyzed as described (*10*), except that counts were determined with a Betascope (Betagen). Affinities were estimated from binding curves as that amount of repressor (R) giving half-maximal protection (*12*). Experiments at 150 and 200 mM KCl with intact LexA gave an affinity for the intact operator of 1.5 and 2.5 nM, respectively; for half-operator we did not reach 100% occupancy but estimated that the affinity was ≈2 and 3 μM, respectively. At 100 mM KCl, from $K_{app} = 0.5 \times 10^{-18}$ M^2 and $K_{dimer} = 15$ μM (*28*), we calculate that the value of K_d in pathway I (Fig. 1A) was $\approx 3 \times 10^{-14}$ M. Tighter binding of the NH_2-terminal fragment to the *recA* operator has been observed under somewhat different conditions (*29*). We cannot account for this discrepancy. It is unlikely to result from differences in the protein preparation, as we obtained the same affinity with a protein prepared by autodigestion of LexA at pH 9.0 for 4 hours (*29*), and the affinity was essentially the same at pH 7.4 and 8.0.

KCl (mM)	I R	I NH_2 terminal R	HO R	HO NH_2 terminal R
	[LexA] giving 50% protection (nM)			
50	0.40	400	350	450
100	0.70	750	650	750

Binding of the second LexA monomer involves two steps, protein-protein interactions and protein-DNA interactions, which could proceed in either order (Fig. 1B). In either pathway IIa or IIb, relatively little entropy should be lost in the second, unimolecular step (*19, 20*); hence, these steps should be more favorable than their bimolecular counterparts (*21*). Other proteins could bind as tightly as LexA to intact operator but give weaker half-site binding for either of two reasons. First, the cooperativity for binding the second monomer could be greater than for LexA (*21*). Second, the structure of the protein might change upon binding to DNA (*3, 22*), allowing additional protein-protein contacts to form, or stabilizing a conformation disfavored in solution.

More generally, binding of the second LexA monomer can serve as a model system for the assembly of DNA-bound complexes that contain several different factors. Examples of such complexes include pro-

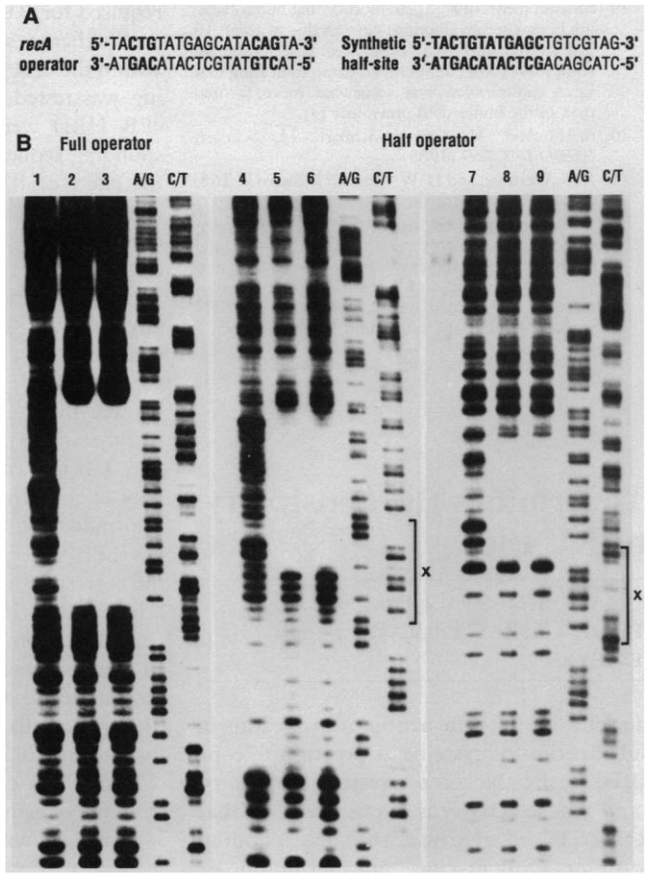

Fig. 2. Binding of LexA to a synthetic operator half-site. (**A**) Sequence of the *recA* operator and a synthetic half-site corresponding to the left half of this operator. In the *recA* operator, nucleotides most highly conserved among LexA binding sites (*30*) are shown in bold type. Sequences in the central portion of the operator are not conserved. In the half-site, bold type indicates the region identical to the *recA* operator. Sequences replacing the right portion of the operator are also shown. (**B**) DNase I footprinting was used to show binding to the intact operator and the operator half-site by intact LexA and its NH_2-terminal domain. Lanes 1 to 3 are the intact operator labeled in the top strand, as shown in (A); lanes 4 to 6 are the half-operator labeled at the 3′ end of the top strand; lanes 7 to 9 are the half-operator labeled at the 5′ end of the bottom strand. Lanes 1, 4, and 7, no protein; lane 2, 2.5 nM intact LexA; lanes 5 and 8, 2.5 μM intact LexA; lanes 3, 6, and 9, 2.5 μM NH_2-terminal fragment. The positions of sequences replacing the other half of the *recA* operator are indicated by "X". Footprinting buffer (*25*) contained 20 mM tris · HCl, pH 7.4, 10% sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 1.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% bovine serum albumin, and KCl and DNA as indicated (*11*).

karyotic replication complexes, site-specific recombination complexes, the general transcription machinery of eukaryotes, and the interaction of general transcription factors with transcriptional activator proteins.

Many fusion proteins between the LexA DNA-binding domain and the transcription activation domains of various eukaryotic regulatory proteins can activate transcription (*23*). Although some of these transcriptional regulatory proteins, such as GCN4 and GAL4, are known to dimerize (*24*), it is unlikely that all of these fusion proteins can readily dimerize (*25*), either because the activator protein lacks this capability or because the portion fused to LexA does not provide dimer contacts. We suggest that dimerization may not be necessary to achieve DNA binding and transcription activation. Certain fusion proteins may bind to DNA as monomers if they are present in the nucleus at a concentration that approaches 1 μM. Because activation assays can detect a low fractional occupancy of DNA binding sites, a relatively modest affinity might suffice, if a single monomer of bound activator can activate transcription.

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12. In both pathways, the overall reaction is $2\,M + O \rightleftharpoons M_2O$ (M, monomer; O, operator), and the dissociation constant is $K_{app} = [M]^2 \cdot [O]/[M_2O]$, in units of M^2. Hence, the measured value of [LexA] at 50% protection (where [O] = [M_2O], Table 1) is $K_{app}^{1/2}$. In pathway I, K_{app} is the product of the dissociation constant of the dimer binding to the operator (K_d) and the dissociation constant for monomer-dimer interconversion (K_{dimer}); $K_{app} = K_d \cdot K_{dimer}$. In pathway II, $K_{app} = K_{m1} \cdot K_{m2}$. $K_{app}^{1/2}$ and K_{m1} are the measured affinities for intact operator and half-operator, respectively (Table 1), and we calculated K_{m2} using the equation just given. Because the cycle is closed, the K_{app}'s for the two pathways have the same value. Cooperativity for binding the second monomer is given by the ratio of association constants $(1/K_{m2})/(1/K_{m1}) = K_{m1}/K_{m2} = K_{m1}^2/K_{app}$.
13. Three lines of evidence suggest that a nonspecifically bound subunit would give a footprint. First, the complex of glucocorticoid receptor with an altered binding site contains one monomer bound nonspecifically (*16*), and this monomer makes numerous contacts with the nonspecific site. Second, we have found that high concentrations of coliphage HK022 repressor protect nonspecific sites, adjacent to specifically bound repressor molecules, from DNase I (N. G. Carlson and J. W. Little, unpublished data). Third, *Acanthamoeba* RNA polymerase I binds nonspecifically to sites adjacent to the TIF factor and makes a DNase I footprint [P. Kownin, E. Bateman, M. R. Paule, *Cell* **50**, 693 (1987)].
14. Labeled DNA (2 μl) was mixed with LexA (1 ml), and aliquots were treated with DNase I for 10 s at intervals. The same extent of protection was seen when DNase was added at 10 s as at later times, either at 50 or 100 mM NaCl with 400 or 800 pM LexA, respectively.
15. The rate of complex formation from free dimers is too slow, even granting two assumptions favorable to pathway I: that the forward rate constant, k_f, for LexA dimers binding to DNA is as rapid as with Lac repressor (*28*) (≈10^{10} M^{-1} s^{-1}), and that the concentration of dimers would not be depleted by DNA binding, as a result of rapid equilibration with monomers. In 100 mM KCl, 800 pM LexA contains 0.04 pM dimers, given a K_{dimer} of 15 μM (*29*). With these values, the initial rate of operator:dimer formation, given by $d[D:O]/dt = k_f \cdot [O] \cdot [D]$ (D is a dimer), is equal to 0.0004 [O]/s; that is, 0.04% of the operators bind to dimers per second, far too slow to bind 60% of the operators within 20 s.
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21. To estimate the equilibrium constants in these pathways, we assumed that in pathway IIa the second monomer binds with the same affinity as the first ($K_{1a} = K_{m1} \approx 0.7$ μM at 100 mM KCl) and that in pathway IIb K_{1b} has the same value as for solution dimerization (≈10 to 20 μM). Because $K_{m2} = K_{app}/K_{m1} \approx 0.7 \times 10^{-12}$ M, the second, unimolecular steps would have dissociation constants $K_{2a} \approx 1.5 \times 10^{-6}$ and $K_{2b} \approx 0.5 \times 10^{-7}$ to 1×10^{-7}, respectively, because $K_{1a} \cdot K_{2a} = K_{1b} \cdot K_{2b} = K_{m2}$. Hence, the effective concentration of the bound second monomer for the first monomer or for the DNA, respectively, [K_{1b}/K_{2a} or K_{1a}/K_{2b} (*20*)], is roughly 10 M; in other systems (*2*) this value could be far higher (*19, 20*), yielding higher cooperativity parameters.

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A Single Amino Acid That Determines the Sensitivity of Progesterone Receptors to RU486

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The progesterone analog RU486, an abortifacient, inhibits the action of progestins in humans but not in chickens or hamsters. Substitution of cysteine at position 575 by glycine in the hormone binding domain (HBD) of the chicken progesterone receptor (cPR) generated a cPR that binds RU486 and whose activity is antagonized by that compound. In fact, all receptors that bind RU486 have a glycine at the corresponding position. The hamster PR, like cPR, has a cysteine. Only glycine—not methionine or leucine—at position 575 allowed binding of RU486 to cPR. Substitution of this glycine by cysteine in the human PR (hPR) abrogated binding of RU486 but not that of an agonist. The corresponding mutation in the human glucocorticoid receptor resulted in a loss of binding of both dexamethasone and RU486. Examination of a series of 11β-substituted steroids showed that antagonism is not an intrinsic property of an antihormone, because one hPR antagonist acted as an agonist for a mutated hPR. The positioning of an aromatic 11β-substitution in the PR HBD appears to be critical for generating agonistic or antagonistic activity.

UNDERSTANDING HOW AGONISTS and antagonists interact with their receptors is important for the design of pharmaceuticals. RU486 competitively binds to the progesterone receptor (PR) of certain species and inhibits progestin action (*1*). The PR is a member of the nuclear receptor family (*2*) and its DNA binding domain (DBD) and hormone-binding domain (HBD) have been identified (*3, 4*), as well as two transcription activation functions (TAFs), in the NH_2 terminal region A/B (TAF-1) and in the HBD (TAF-2) (*5*) (Fig. 1B). Binding of RU486 induces PR dimerization and DNA binding in vivo and in vitro (*5, 6*), but no activity of TAF-2 was observed in the presence of RU486 (*5*). In contrast to the human PR (hPR), both chicken (cPR) and hamster (haPR) PRs do not bind RU486. But several agonists bind similarly to all of these PRs. Moreover, RU486 inhibits the activity of the glucocorticoid receptor in both humans and chickens (*1*).

Regions other than the HBD do not appear to contribute to RU486 binding by hPR, because a fusion protein composed of β-galactosidase and the hPR HBD (amino acids 687 to 933) bound the antagonist with an affinity similar to that of the complete human PR. Binding of RU486 by a fusion protein containing the corresponding portion of cPR was barely detectable (7) (Fig. 2D). This result indicates that the 31 amino acids that differ in the HBDs of cPR and hPR are responsible for the differential RU486 binding and that no post-translational modification specific to eukaryotes is required for RU486 binding.

Whether a specific region (or regions) within the cPR HBD blocked RU486 binding was tested as follows. Portions of the cPR HBD were replaced with the corresponding sequences of hPR (Fig. 1B), and the effect of RU486 on the ability of the chimeras to induce transcription in the presence of agonist (RU27987) was investigated (Fig. 1A) by transiently transfecting HeLa cells with a reporter gene (MMTV-CAT) carrying the progestin responsive element (PRE) of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (*8*). RU486 is a complete hPR antagonist in this system (*5*). All chimeras (m1 to m4; Fig. 1B) induced transcription in the presence of the agonist, but only the transcription induced by RU27987 and m2, which has the hPR sequence 716 to 766 in place of the corresponding sequence of cPR (amino acids 569 to 619), was inhibited by RU486 (Fig. 1C). Immunoblots demonstrated that all chimeras were expressed at similar concentrations; thus, they activated transcription with similar efficiencies, indicating that the sequence differences within the HBDs of cPR and hPR do not significantly affect the structure or activity of TAF-2 or that TAF-2 is contained within one of the transposed sequences.

The segment of the PR HBD present in cPR m2 differs at six residues from the corresponding sequence of cPR (Fig. 3A). Two of these residues are conserved in hPR and human glucocorticoid receptor (hGR), both of which bind RU486 (hPRGly^{722}, hGRGly^{567}; hPRLeu^{763}, hGRLeu^{608}). The rabbit PR (rbPR), which also binds RU486, has a sequence identical to the hPR HBD sequence present in m2 (Fig. 3, A and B). We therefore analyzed the effect of replacements of these two residues with the corresponding amino acids from the hPR (*9*). Whereas cPR(Met^{616}-Leu) still behaved like cPR (Fig. 1D), RU27987-induced transcription by the double mutant cPR(His^{573}-Gln, Cys^{575}-Gly) was inhibited by RU486 (Fig. 1D). This sensitivity to RU486 was solely due to the mutation of cPRCys^{575} to Gly, because cPR(His^{573}-Gln) behaved like wild-type cPR (Fig. 1, C and D), whereas RU486 efficiently inhibited transcription induced by cPR(Cys^{575}-Gly) in the presence of RU27987 (Fig. 1D). Analysis of RU486 binding to bacterially expressed β-galactosidase fusion proteins confirmed that the HBDs of m2 and cPR(Cys^{575}-Gly) bound RU486 (Fig. 2D). That these two chimeras bound RU486 with an affinity lower than that of hPR indicates that additional divergent amino acids contribute to the binding of RU486 by hPR. However, the single amino acid

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