Table 5. Effect of divalent cations on the complexation of [^{125}I]hGH to hPRLbp (at 60 or 5000 pM) at physiological total serum concentrations (*17*). Highly purified metal ion salts for $CaCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$ were obtained from Johnson-Matthey Purotonic, Sigma, Mallinckrodt, Mallinckrodt and Johnson-Matthey Purotonic, respectively. Binding assays were performed as described in Fig. 2 in 0.1% BSA, 140 mM NaCl, 20 mM tris (pH 7.5) at 25°C. The percentage of complex formed was calculated from the ratio of the amount of [^{125}I]hGH·hPRLbp complex immunoprecipitated to total [^{125}I]hGH present in the assay.

Divalent metal (concentration)	Complex formation (%)	
	60 pM	5000 pM
None	3.8 ± 1.4	5.4 ± 0.8
Ca^{2+} (5 mM)	1.3 ± 0.7	29.0 ± 1.4
Co^{2+} (5 nM)	1.3 ± 2.4	6.8 ± 1.5
Cu^{2+} (20 μM)	1.7 ± 1.5	29.0 ± 1.4
Mg^{2+} (2 mM)	0.8 ± 1.4	9.1 ± 3.6
Mn^{2+} (2 μM)	0.4 ± 0.8	8.2 ± 1.8
Zn^{2+} (20 μM)	46.0 ± 3.0	74.0 ± 1.0

involvement of zinc in hormone action by showing that it mediates directly the interaction between a polypeptide hormone and an extracellular receptor. The growth hormone and prolactin receptors are homologous to receptors of the cytokine super family (*24*) which includes the interleukin-2 (IL-2), IL-3, IL-4, IL-6, IL-7, erythropoietin, and GM-CSF receptors. It is possible that zinc may mediate other hormone-receptor interactions such as these.

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7. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His, I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Stable, Monomeric Variants of λ Cro Obtained by Insertion of a Designed β-Hairpin Sequence

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λ Cro is a dimeric DNA binding protein. Random mutagenesis and a selection for Cro activity have been used to identify the contacts between Cro subunits that are crucial for maintenance of a stably folded structure. To obtain equivalent contacts in a monomeric system, a Cro variant was designed and constructed in which the antiparallel β-ribbon that forms the dimer interface was replaced by a β-hairpin. The engineered monomer has a folded structure similar to wild type, is significantly more stable than wild type, and exhibits novel half-operator binding activity.

ONE TEST OF OUR UNDERSTANDING of protein structure is the design of analogs of minimal complexity that retain the ability to fold as they would in the intact protein (*1*). Although dimers of λ Cro are composed of two distinct globular domains (*2*), contacts from both subunits are required for stability, since dissociation of the wild-type (wt) dimer is normally accompanied by denaturation (*3*). In this report we identify the intersubunit contacts that are required for Cro stability and describe the construction and characterization of an engineered Cro variant in which the connectivity of the polypeptide chain is altered to maintain these contacts in a stably folded monomer. This engineered monomer defines a simplified folding unit that retains the basic structural features of Cro.

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As shown in Fig. 1, A and B, the dimer interface of Cro consists of a β-strand from the carboxyl-terminus of each subunit, which pair to form an antiparallel β-ribbon (*2*). Residues 52 to 58 from each subunit form this structure and account for most of the intersubunit contacts. We probed the importance of the side chains at these positions by randomization using cassette mutagenesis (*4*) followed by a biological selection for Cro-mediated repression. The active sequences that were obtained are shown in Fig. 2A. Substantial sequence variability is allowed at every position except 58, where only the wt Phe and the conservative Tyr substitution are tolerated. These results suggest that the nature of the side chain at position 58 is critical for Cro function, whereas the identities of the side chains at positions 52 to 57 are less important. In the crystal structure (*2*), the Phe^{58} side chain (colored red in Fig. 1B) from one subunit packs into a crevice on the other subunit,

forming part of the hydrophobic core and making extensive van der Waals contacts with surrounding side chains. It seems plausible that the loss of these Phe[58]-mediated contacts, upon dissociation of the dimer, is the key factor that causes concomitant unfolding of the globular portion of the Cro monomer. The main-chain hydrogen bonds between the strands of the antiparallel β-ribbon are probably also important in stabilizing Cro, but these interactions could be formed in any of the mutant interfaces.

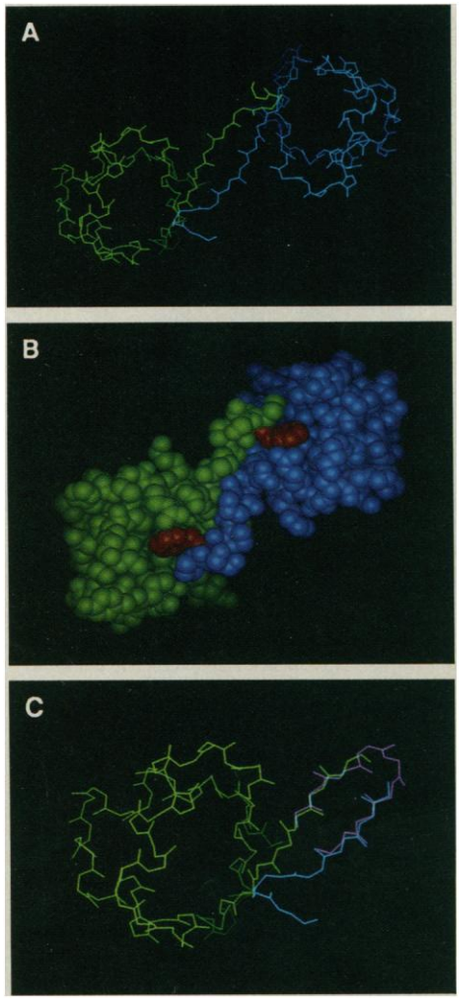

Fig. 1. λ Cro structures. (**A**) Polypeptide backbone of the Cro dimer. Residues 1 to 60 are shown in green for the O subunit and in blue for the B subunit. (**B**) Space-filling representation of the Cro dimer. Phe58 from each subunit is colored red. (**C**) Backbone tracing of a potential Cro monomer. Residues 1 to 56 of the O subunit and 54 to 60 of the B subunit are shown with a type I′ turn (in purple) from superoxide dismutase (SOD) (*11*) (residues 21 to 28 EAKGDTVV) (*12*) superimposed on the dimer interface of Cro. The superposition was carried with the Insight program (Biosym Inc.). The SOD residues 21 to 23 (EAK) and 26 to 28 (TVV) were aligned with Cro subunit O residues 54 to 56 (EVK) and subunit B residues 54 to 56 (EVK), respectively. The root-mean-square (rms) deviation of the aligned backbone structures was 0.77 Å. Cro coordinates were provided by D. Tronrud and B. Matthews. Cro residues 61 to 66 are not shown in any of the panels as they are not well ordered in the crystal structure.

In designing potentially stable monomeric variants of Cro, it seemed important to maintain the Phe[58] packing contacts and as many of the β-sheet hydrogen bonds as possible (Fig. 2B). One simple way to do this is illustrated schematically in Fig. 2C. Overall, five residues are inserted into the Cro sequence; the first two residues (L1 and L2) should form a β-turn, with the next three residues forming a β-strand that maintains the register of hydrogen-bonded structure in the hairpin. This design seemed reasonable on steric grounds as a type I′ β-turn from the compendium of Sibanda *et al.* (*5*) could be superimposed on the Cro structure (Fig. 1C).

To maximize the chances of obtaining an appropriate hairpin turn, we synthesized a cassette that was degenerate at the L1 and L2 positions (Fig. 2C) and would consequently generate all consensus type I′ and II′ turns. These two turn types are the most commonly observed in tight β-hairpins (*5*). This cassette was introduced into the *cro* gene, and 50 unselected transformants of *Escherichia coli* were picked and the corresponding genes were sequenced. Clones with 16 different L1-L2 sequences were recovered. Protein levels in cell lysates from each clone were assayed by SDS gel electrophoresis. These levels provide a rough indication of protein stability, since unstable Cro mutants are rapidly degraded (*3, 6*). Among the sequences that resulted in moderate to high levels of protein were the three consensus type I′ turn sequences (DG, GG, and NG) and the two type II′ consensus sequences (GS and GT). Three additional sequences (GA, NS, and DA) also gave reasonable levels of protein, while the remaining nonconsensus sequences (DT, ET, KS, KT, NT, RT, SS, and ST) resulted in low protein levels.

We picked one of the most stable candidate proteins (designated Cro.mDG, with Asp and Gly at the L1 and L2 positions, respectively) to examine in detail. The DG protein behaved much like wt Cro during purification (*3*), but bound somewhat less tightly to phosphocellulose and eluted at a lower apparent molecular weight from a gel filtration column. Sedimentation equilibrium experiments were performed (*7*) to determine the oligomeric state of the protein. The ratio of the observed molecular weight to that calculated from the amino acid sequence was 1.04 ± 0.14 ($n = 2$) for Cro.mDG and 1.9 in a single determination for wt Cro. These results indicate that the DG variant is indeed a monomer whereas wt Cro is a dimer, as expected.

Circular dichroism spectra were determined for Cro.mDG and wt Cro to assess similarities in secondary structure. As shown in Fig. 3A, both proteins have similar spectra, indicating that the basic Cro fold has been maintained in the monomeric variant. As expected for a monomeric protein, the thermal stability of the DG variant was independent of concentration (Fig. 3B). In contrast, the wt melt was markedly concentration dependent, as expected for a protein that denatures in a concerted reaction from a folded dimer to two unfolded monomers. Somewhat surprisingly, the DG monomer

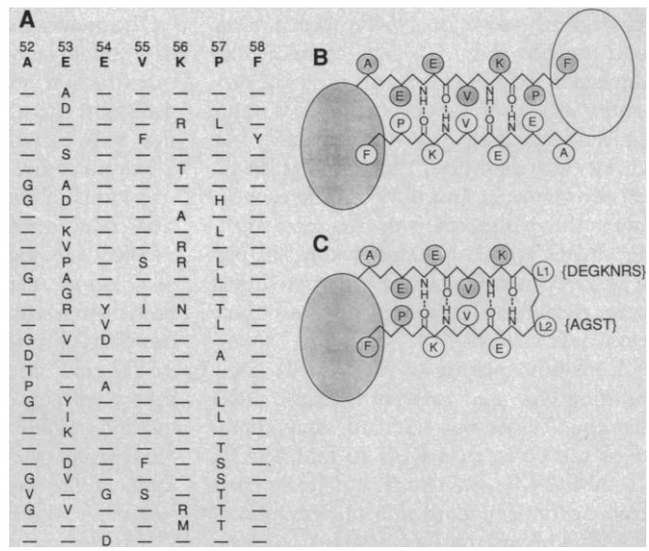

Fig. 2. (**A**) Active sequence variants. The amino acid residues at positions 52 to 58 of wt Cro are indicated in the one letter code (*12*) at the top. Below are listed the sequences from active *cro* genes, isolated following cassette mutagenesis (*13*) (wt residues are indicated by a dash). (**B**). Schematic diagram of the wt interface. The globular portion of each subunit is represented as a large oval. Residues 52 to 58 from each subunit are depicted in an extended configuration. Hydrogen bonds in the dimer interface are indicated. (**C**). Monomer design. In the monomer model the five-residue insertion (open circles) into the wt sequence (shaded circles) consists of two loop positions (L1 and L2) in which the listed set of amino acids were inserted through the use of the degenerate codons (A/G, A/G, A/C) and (A/G, C/G, C), respectively, and a duplication of residues 54 to 56.

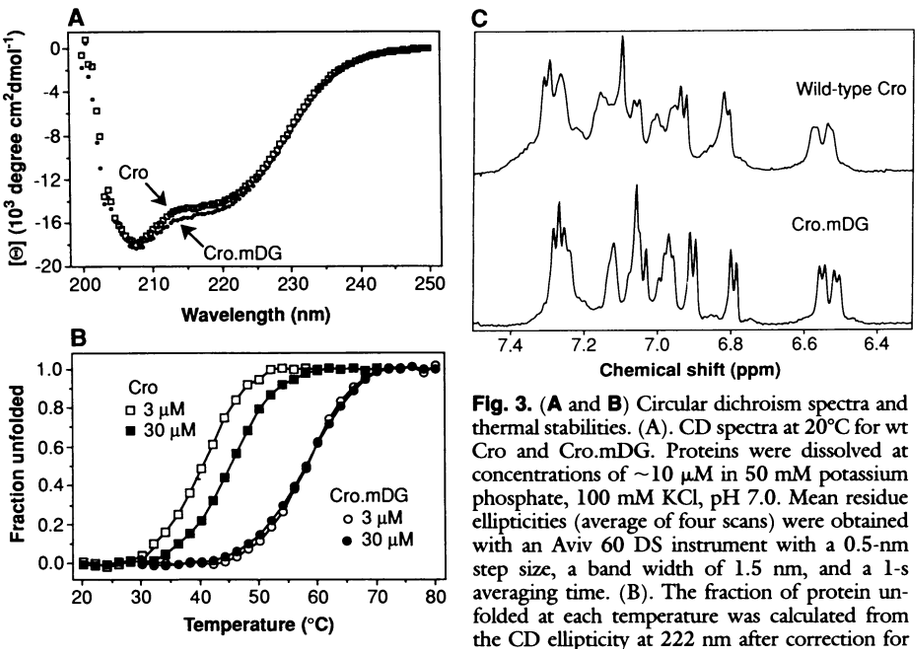

Fig. 3. (**A** and **B**) Circular dichroism spectra and thermal stabilities. (A). CD spectra at 20°C for wt Cro and Cro.mDG. Proteins were dissolved at concentrations of ~10 μM in 50 mM potassium phosphate, 100 mM KCl, pH 7.0. Mean residue ellipticities (average of four scans) were obtained with an Aviv 60 DS instrument with a 0.5-nm step size, a band width of 1.5 nm, and a 1-s averaging time. (B). The fraction of protein unfolded at each temperature was calculated from the CD ellipticity at 222 nm after correction for the pre- and posttransition baselines. Thermal melts were completely reversible for both proteins. Solutions conditions were the same as in (A). (**C**) NMR spectra of the aromatic region for wt Cro and Cro.mDG. The ^1H NMR spectra were taken at 30°C in D_2O) containing 20 mM potassium phosphate, pH 6.8. Protein concentrations were 2.7 mM for the wt protein and 1.4 mM for Cro.mDG. Data were collected on a 500-MHz spectrometer at the Francis Bitter National Magnet Lab at MIT.

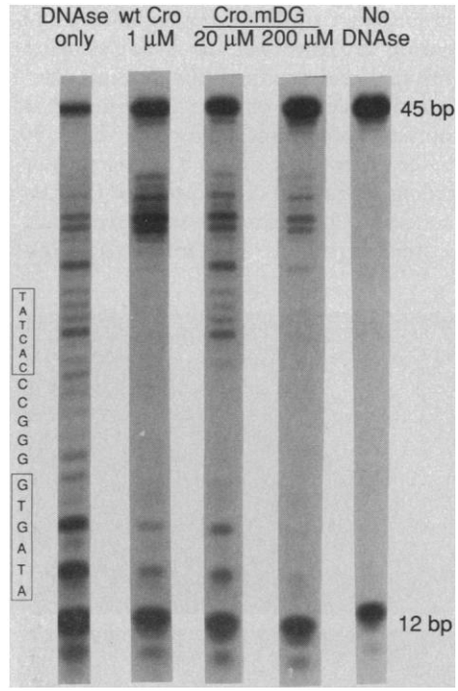

Fig. 4. DNAse I protection assays. The DNAse only lane shows the pattern of bands obtained in the absence of added Cro protein. The no DNAse lane shows undigested DNA (*14*), which consists of the operator-containing fragment (45 bp) and a smaller fragment (12 bp). Other lanes show the digestion patterns obtained in the presence of the specified protein at the indicated concentrations. The position of cleavage products relative to the sequence of the operator site is indicated on the left. Bases specifically recognized by wt Cro (*15*) are boxed. The operator is symmetric except for the central base pair, but the DG protein preferentially bind to one half-site. This result may indicate that the central base or flanking sequences influence binding affinity.

(midpoint temperature, T_m = 58°C) was significantly more stable than wt Cro (T_m = 45°C at the highest concentration tested). We assume that this enhanced stability reflects effective concentrations (*8*) of interacting groups in the monomer, which are significantly higher than the concentrations of the corresponding groups in the bimolecular folding and association of wt Cro molecules.

Further evidence for structural similarity between Cro.mDG and wt Cro was obtained by comparison of the one-dimensional nuclear magnetic resonance (NMR) spectra of the two proteins (Fig. 3C). The chemical shift dispersion of proton resonances in the aromatic region of the two spectra is quite similar, indicating that the aromatic side chains (including Phe^{58}) have similar chemical environments. One obvious difference between the two spectra is that the lines in the Cro.mDG sample are significantly sharper, which we expected since the monomer should experience faster tumbling and thus more efficient relaxation.

Deoxyribonuclease I (DNAse I) footprinting was used to assay operator DNA binding. Under our standard assay conditions (20°C, 50 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$), wt Cro shows nearly complete operator binding at a concentration of 5 nM, whereas the DG protein shows no operator protection at a concentration of 10 μM. Hence, under these conditions, binding of the DG monomer is reduced by at least 2,000-fold. Under low salt and low temperature conditions (0°C, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) (*9*), we observed binding of Cro.mDG to operator DNA (Fig. 4). At a concentration of 20 μM, the DG protein protects only the lower half-site and central region of the operator. At a higher concentration (200 μM) the upper half-site was also protected. This independent binding of Cro.mDG to operator half-sites clearly distinguishes it from wt Cro, which always protects both halves of the operator cooperatively [Fig. 4; also see (*10*)].

The monomeric Cro variants described here should be useful for a number of structure-function studies. One priority is to determine the structure of the monomer, as this will establish whether the expected β-hairpin is present and reveal possible perturbations in the globular portion of Cro. The monomeric Cro.mDG protein is an excellent candidate for NMR determination of solution structure. These studies have been hampered for the wt Cro dimer by problems of solubility and because intramolecular and intermolecular nuclear Overhauser effects cannot be readily distinguished. Comparison of operator DNA binding by the monomeric and dimeric forms of Cro should aid in determining the role of the dimer interface in coupling the DNA binding energies of individual subunits. Finally, comparison of the stabilities and structures of monomeric variants with different β-hairpin sequences should be useful in quantifying the β-turn propensities of these amino acid sequences.

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7. Solutions (100 μM) of wt Cro and Cro.mDG in 50 mM potassium phosphate, pH 7.0, 100 mM KCl were allowed to come to equilibrium at 20,000 rpm in a Beckman AN-G rotor in a model E analytical ultracentrifuge. Ultraviolet absorbance data at 280 nm was collected with a photoelectric scanner and analyzed as described [C. H. Chervenka, *A Manual of Methods for the Analytical Ultracentrifuge* (Spinco, Beckman, Inc., Palo Alto, CA, 1970)]. Partial specific volumes were calculated as the mass average partial specific volume with the values for the individual amino acids of A. A. Zamyatnin [*Prog. Biophys. Mol. Biol.* **24**, 1007 (1972)].
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13. To mutagenize residues 52 to 58 simultaneously, the corresponding codons of one DNA strand of a cassette were synthesized by using a mixture of 62.5% of the wt base and 12.5% of each of the other three bases. This level of mutagenesis resulted in an average of two to three amino acid sequence changes per mutant protein. Synthesis of the second, complementary strand was performed enzymatically with a self-priming strategy [A. K. Oliphant, A. L. Nussbaum, K. Struhl, *Gene* **44**, 177 (1986)]. Double-stranded cassettes were generated by restriction cleavage and ligated into pUCroRS. This plasmid consists of the Eco RI to Sma I fragment of pAP119 (*3*) cloned between the Eco RI and Hinc II sites of pUC18 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)]. Plasmids were transformed into a strain in which Cro-mediated repression of a ribosomal protein s12 gene was required for resistance to streptomycin [M. C. Mossing and R. T. Sauer, in preparation; for an analogous selection, see (*4*)]. Active genes (identified as streptomycin resistant colonies) were recovered in <1% of transformants. The level of activity required for resistance varied depending on the level of Cro expression. When Cro expression was fully induced, <5% of wt activity was required.
14. A pUC19 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)] derivative plasmid containing a synthetic consensus Cro operator between the Xba I and Pst I sites was opened at the Bam H1 site and filled in with α-^{32}P–labeled dNTPs (deoxyribonucleotide triphosphates) by using the Klenow fragment of DNA polymerase. Subsequent digestion with Hind III and Kpn I yielded 12- and 45-bp (operator-containing)–labeled fragments as well as an unlabeled backbone fragment of 2.6 kb. This crude mixture was diluted into low salt buffer (final operator concentration 1 nM) and mixed with Cro or Cro.mDG, incubated for 20 min on ice, and subjected to DNAse digestion as described (*9*).
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Inheritance of Proliferative Breast Disease in Breast Cancer Kindreds

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Previous studies have emphasized that genetic susceptibility to breast cancer is rare and is expressed primarily as premenopausal breast cancer, bilateral breast cancer, or both. Proliferative breast disease (PBD) is a significant risk factor for the development of breast cancer and appears to be a precursor lesion. PBD and breast cancer were studied in 103 women from 20 kindreds that were selected for the presence of two first degree relatives with breast cancer and in 31 control women. Physical examination, screening mammography, and four-quadrant fine-needle breast aspirates were performed. Cytologic analysis of breast aspirates revealed PBD in 35% of clinically normal female first degree relatives of breast cancer cases and in 13% of controls. Genetic analysis suggests that genetic susceptibility causes both PBD and breast cancer in these kindreds. This study supports the hypothesis that this susceptibility is responsible for a considerable portion of breast cancer, including unilateral and postmenopausal breast cancer.

BREAST CANCER HAS LONG BEEN RECognized to be a familial disease (*1*). Population-based studies have shown that a woman's risk of developing breast cancer is increased 1.5- to 3-fold if one first degree relative (mother, daughter, or sister) had breast cancer and 5- to 10-fold if the relative had bilateral cancer or if more than one first degree relative had breast cancer (*2*). The hypothesis of a rare breast cancer–susceptibility allele that would be dominantly inherited has been supported by several studies (*3–6*) that have produced estimates of the gene frequency of a dominant susceptibility allele of 0.0006 to 0.008 in the population and a lifetime probability of breast cancer of 0.57 to 0.92 in genetically susceptible individuals. The following analysis suggests that genetic predisposition to breast cancer may be more common in postmenopausal breast cancer than previously thought and that this predisposition is expressed as PBD.

Proliferative breast disease has been used to describe several benign breast lesions characterized by excessive but nonmalignant proliferation of breast epithelial cells located in the terminal portions of the ducts of the breast (*7–8*). It has been suggested that PBD represents a premalignant state, because (i) breast cancer most often originates in the terminal ductal-lobular unit, (ii) proliferative lesions exhibit a spectrum of morphologic changes, the most severe of which resemble non-invasive cancer, and (iii) foci of PBD are often found in breast cancer mastectomy specimens (*9*). Several cohort studies have demonstrated that women with PBD have a two- to fivefold increased risk of developing breast cancer when compared to women with nonproliferative breast lesions (*10–12*). A family history of breast cancer in addition to the presence of PBD further increases the risk of breast cancer (*11*). In these studies, PBD was detected when women underwent surgical biopsy of clinically suspicious lesions.

We examined the frequency of PBD in families that were ascertained by two first degree relatives with breast cancer and tested the hypothesis that PBD and breast cancer are inherited lesions in these families (Fig. 1). All kindreds meeting this criteria were ascertained from the hematology/oncology clinic at the University of Utah, from the private clinic of one of the authors (H.H.), and the Utah Family Health Tree Project (*13*). One kindred, K1900, was selected with a third diagnosed individual (proband). All probands were classified as premenopausal or postmenopausal. If menopausal status was unknown, women diagnosed with breast cancer at age 50 or less were classified as premenopausal. The probands had premenopausal breast cancer in five kindreds and postmenopausal breast cancer in seven kindreds; seven kindreds had both one premenopausal and one postmenopausal proband. K1900 had one premenopausal and two postmenopausal probands. Nineteen kindreds were of

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