$$r_t = f_{obs}/0.987\, f_D$$

where $f_{obs} = P_{obs}/S_0$. The corresponding correction for r_t at t_∞ is

$$r_\infty = f_{obs}/0.987\, f_D = 1/0.987$$

The final expression for the D/T isotope effect is

$$k_D/k_T = \frac{\ln(1 - f_D)}{\ln\{1 - f_D[\frac{(^3H/^{14}C)_f\, r_t}{(^3H/^{14}C)_\infty r_\infty}]\}} = \frac{\ln(1 - f_D)}{\ln\{1 - f_{obs}[\frac{(^3H/^{14}C)f}{(^3H/^{14}C)_\infty}]\}}$$

29. If hydrogen-isotope effects result only from differences in zero-point energy, then the following equation can be derived

$$\frac{\ln(k_D/k_T)}{\ln(k_H/k_T)} = \frac{1/(m_D)^{1/2} - 1/(m_T)^{1/2}}{1/(m_H)^{1/2} - 1/(m_T)^{1/2}}$$

The factor on the right-hand side is 3.26 for $m_H{:}m_D{:}m_T$ in the ratio of 1:2:3, or 3.34 if they are in the ratio of the reduced masses of ^{12}C–H:^{12}C–D:^{12}C–T. See (7).

30. J. L. Hogg, in *Transition States of Biochemical Processes*, R, D. Gandour and R. L. Schowen, Eds. (Plenum, New York, 1978), pp. 201–224.
31. Cleland and co-workers have reported that secondary H/D isotope effects with formate dehydrogenase were reduced from 1.23 ± 0.03 to 1.07 ± 0.02 when substrate oxidation involved C–D as opposed to C–H bond cleavage. This result was attributed to a combination of coupled motion and tunneling. See J. D. Hermes and W. W. Cleland, *J. Am. Chem. Soc.* **106**, 7263 (1984), and J. D. Hermes, S. W. Morrical, M. H. O'Leary, W. W. Cleland, *Biochemistry* **23**, 5479 (1984).
32. R. A. Marcus, *J. Phys. Chem.* **72**, 891 (1968).
33. As discussed by M. J. Stern and R. E. Weston, Jr. [*J. Chem. Phys.* **60**, 2808 (1973)], A_H/A_D is expected to be quite temperature-dependent for reactions that include a tunneling correction, leading to extrapolated values that are either significantly reduced or greater than unity. In the low-temperature limit such that tunneling dominates the reaction-coordinate motion for H, D, and T, $A_H/A_D >> 1$.
34. The relation between H/T and D/T isotope effects in the temperature range of 0° to 40°C is similar to that shown in Table 2.
35. E. Cedergren-Zeppezauer, J. P. Samama, H. Eklund, *Biochemistry* **21**, 4895 (1982).
36. H. Eklund, B. V. Plapp, J. P. Samama, C-I. Branden, *J. Biol. Chem.* **257**, 14349 (1982).
37. J. T. Bolin, D. J. Filman, D. A. Matthews, R. C. Hamlin, J. Kraut, *ibid.*, p. 13650.
38. From x-ray data, the distance between the C-4 of cofactor and the C-1 of substrate is 3.5 Å. Using the C–H bond length of 1.1 Å, and the assumption that C–H---C is linear at the transition state, the transferred hydrogen must travel 1.3 Å from reactant to product, and would travel further for a nonlinear transition state. See F. Freund, *Trends Biochem. Sci.* **6**, 142 (1981).
39. Y. Cha, C. J. Murray, J. P. Klinman, unpublished results.
40. Supported by a grant from the National Science Foundation.

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Receptor and Antibody Epitopes in Human Growth Hormone Identified by Homolog-Scanning Mutagenesis

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A strategy, termed homolog-scanning mutagenesis, was used to identify the epitopes on human growth hormone (hGH) for binding to its cloned liver receptor and eight different monoclonal antibodies (Mab's). Segments of sequences (7 to 30 residues long) that were derived from homologous hormones known not to bind to the hGH receptor or Mab's, were systematically substituted throughout the hGH gene to produce a set of 17 chimeric hormones. Each Mab or receptor was categorized by a particular subset of mutant hormones that disrupted binding. Each subset of the disruptive mutations mapped within close proximity on a three-dimensional model of hGH, even though the residues changed within each subset were usually distant in the primary sequence. The mapping analysis correctly predicted those Mab's which could or could not block binding of the receptor to hGH and further suggested (along with other data) that the folding of these chimeric hormones is like that of hGH. By this analysis, three discontinuous polypeptide determinants in hGH—the loop between residues 54 and 74, the central portion of helix 4 to the carboxyl terminus, and to a lesser extent the amino-terminal region of helix 1—modulate binding to the liver receptor. Homolog-scanning mutagenesis should be of general use in identifying sequences that cause functional variation among homologous proteins.

HUMAN GROWTH HORMONE (HGH) PARTICIPATES IN THE regulation of normal human growth and development. This 22-kilodalton pituitary hormone has a multitude of biological effects, including linear growth (somatogenesis), lactation, activation of macrophages, and insulin-like and diabetogenic effects (*1*). Growth hormone deficiency in children leads to dwarfism, which has been successfully treated by injection of hGH. In addition to its biological properties, the antigenicity of hGH is useful for distinguishing genetic and post-translationally modified variants of hGH (*2*), for characterizing any immunological responses to hGH when it is administered clinically, and for quantifying the hormone during circulation.

Human growth hormone is a member of a family of homologous

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hormones that includes placental lactogens, prolactins, and other genetic and species variants of growth hormone (*3*). Among these, hGH alone exhibits broad species specificity and binds monomerically to either the cloned somatogenic liver (*4*) or prolactin receptor (*5*). The gene for hGH has been expressed in a secreted form in *Escherichia coli* (*6*), and the three-dimensional folding pattern for porcine growth hormone (pGH) has been solved at moderate resolution and refinement (*7*). However, little is known of the structural features that modulate binding to these receptors or that define the antigenic sites on the hormone.

Using a stategy we term homolog-scanning mutagenesis, we have mapped regions of the hormone involved in binding to the cloned liver receptor and eight different anti-hGH monoclonal antibodies (Mab's to hGH). Segments of hGH were systematically replaced with analogous sequences from homologs of hGH that have greatly reduced affinity for the receptor and the Mab's. We substituted homologous sequences because genetically related proteins (despite large sequence divergence) have similar three-dimensional structures (*8*). Thus, it seemed likely that homologous sequence substitutions may be more readily accommodated without gross disruption in the native conformation of the molecule. Moreover, mutations that disrupt the native folding of the protein are likely to lead to its degradation by proteases in vivo (*9*). Thus, by homolog-scanning mutagenesis, we expressed stably a set of 17 chimeric hormones that collectively altered 85 out of 191 residues in hGH. Such a small set of mutant hormones was readily analyzed by binding assays, thus obviating the need to presort a library of random mutants by a genetic screen or selection. The antibody and receptor binding site data suggest that these mutations did not cause global changes in the folding of the hormone.

Homolog-scanning mutagenesis. Human placental lactogen (hPL), porcine growth hormone (pGH), and human prolactin (hPRL) were used to make chimeric hormones with hGH because homologs of these hormones have reduced binding affinities (more than 1000 times less) for the human liver hGH receptor (*10*). These hormones differed in sequence from hGH by 15, 32, and 77 percent, respectively (Fig. 1), and therefore provided a wide range of homologous sequences to sample.

Six regions in the hGH sequence were targeted for homolog-scanning mutagenesis (Fig. 1, regions A to F). These segments were separated either by disulfide bonds, by borders of secondary structure, by areas of sequence identity among the hormone family, or by regions that previous experiments suggested were not involved in receptor binding. For example, deletion of the NH_2-terminal 13 amino acids by mutagenesis (*11*) or a natural variant of hGH that deletes residues 32 to 46 (*12*) have been reported to cause relatively small reductions in affinity for the somatogenic receptor. In addition, two-chain derivatives of hGH that were produced by limited proteolysis of hGH and which delete some or all of the residues between 134 and 149 do not significantly affect binding to the liver receptor (*13*).

Initially, a random hybrid library containing various NH_2-terminal lengths of hGH linked to the remaining COOH-terminal portion of pGH was constructed by random recombination of tandemly linked genes (*14*). However, of seven hGH-pGH hybrids whose crossover points were distributed evenly over the hGH gene, only the extreme COOH-terminal hybrid [hGH(1–163) followed by pGH(164–191)] was secreted from *E. coli* in amounts sufficient for purification and analysis.

Helix wheel projections (Fig. 2) derived from the pGH folding model (*7*) show that three of the four helices (helices 1, 3, and 4) are amphipathic with strong hydrophobic moments (*15*), while helix 2 is very hydrophobic. The hydrophobic core in proteins is tightly packed (*16*) and mutations in such buried positions are generally destabilizing (*17*). In fact, the COOH-terminal hGH-pGH hybrid that was weakly expressed introduced four substitutions (M170L, V173A, F176Y, and V180M) that are located on the hydrophobic face of helix four. To circumvent problems with expression, we designed most of the homologous sequence exchange in the helical regions of A, D, E, and F to avoid mutations of residues on the hydrophobic face of the helices. Thus, the mutant of hGH with the pGH sequence from 167 to 181 was designed to retain M170, V173, F176, and V180 (Table 1), which are inside or bordering the hydrophobic face of helix 4. Complete sequence exchanges were constructed in the loop structures in regions B, C, D, E, and F. In several cases an additional charged substitution was either introduced or eliminated to more stringently probe a region. For instance, hPL(46–52) contained an additional Q49E mutation, and hPRL(12–33) contained an additional E30Q mutation.

To facilitate efficient mutagenesis, we assembled a synthetic hGH gene that had 18 different unique restriction sites evenly distributed without altering the hGH coding sequence (*18–20*). This gene allowed insertion of mutagenic cassettes (*21*) or permitted restriction-selection against one of the singly occurring restriction sites after site-specific mutagenesis in a single-stranded vector (*22*). Transcription of the mutant or wild-type genes was controlled by the alkaline phosphatase promoter, and secretion of mature hGH into the periplasm was directed by the heat-stable enterotoxin signal

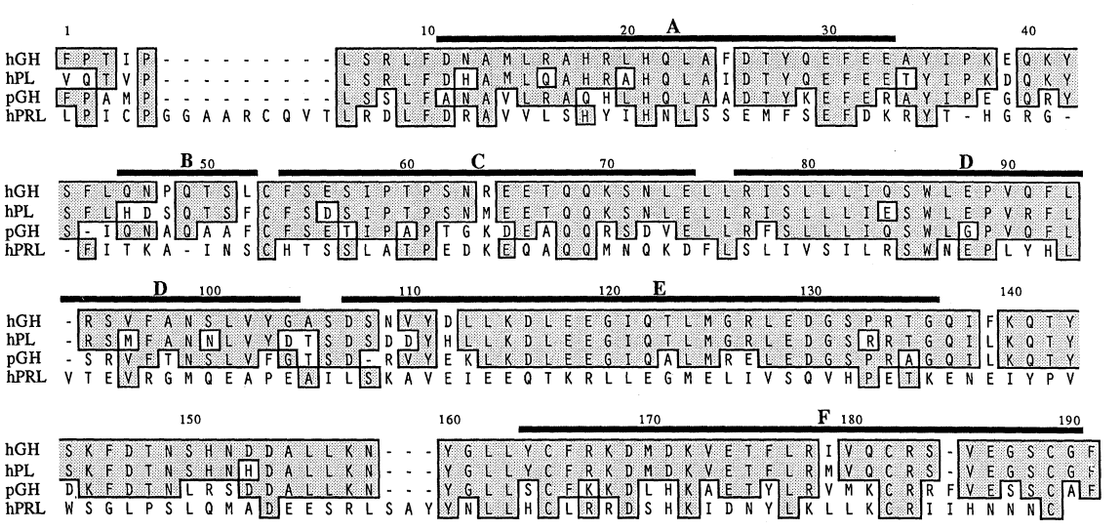

Fig. 1. Sequence alignment of hGH, hPL, pGH, and hPRL. Residues identical to hGH are shown in shaded boxes. Six regions (A to F) where homologous sequences were exchanged are designated above the alignment. Sequences were taken from (*3*) and references as therein. (For single letter amino acid abbreviations, see Table 1.)

sequence (*6*). The receptor and antigenic properties of wild-type hGH produced from the synthetic gene were indistinguishable from hGH produced by the natural cDNA (*19*). Seventeen mutants of hGH containing homologous sequences were constructed (Table 1). Secretion of these chimeric hormones into the periplasmic space of *E. coli* was comparable to that of the wild type and far greater than that of the hGH-pGH hybrids.

Binding of receptor and monoclonal antibodies to chimeric hormones. Competitive binding of ^{125}I-labeled hGH to the extracellular portion of the cloned liver hGH receptor (*4*) was used to quantify the relative affinities of the purified segment substituted growth hormones to the extracellular domain of the liver receptor (Table 1). This receptor fragment (translated molecular size, ~28 kD) was highly purified (*23*), thus avoiding potential artifacts associated with binding to receptors on whole cells. Furthermore, the truncated receptor is highly soluble, exhibits the same selectivity for hGH as the full-length membrane-spanning form (*4, 23*), and retains high binding affinity for hGH (K_D = 0.3 n*M*).

All of the variant hormones having mutations within regions C and F and some within region A caused reductions (tenfold or greater) in binding affinity, whereas those within regions B, D, and E caused relatively small or no changes (Fig. 3). The mutations most disruptive to binding within regions A, C, and F were further localized. Dissection of the hPRL(12–33) into NH_2- and COOH-terminal segments, hPRL(12–19) and hPRL(22–33), respectively, showed that the mutations in the NH_2-terminal portion of region A caused the reduction in binding. The slightly greater reduction caused by the hPRL(12–19) compared to hPRL(12–33) is within the maximal errors (±20 percent) inherent in these measurements (Table 1). Similarly, the NH_2-terminal portion of region F [pGH(167–181)] accounted for a large proportion of the reduction in binding free energy caused by the pGH(164–190) mutant (*24*). One of the most dramatic effects was the 30-fold reduction in binding free energy caused by hPL(56–64), which introduced only two substitutions, E56D and R64M (*24*). Even though the mutants that caused the greatest reductions in binding affinity are in regions widely separated in primary sequence, together these define a patch on the folded hormone that includes the NH_2-terminus of helix 1, the loop from Cys^{53} to the start of helix 2, and the central portion of helix 4 (Fig. 4).

An enzyme-linked immunosorbent assay (ELISA) was used to assess the binding of eight different monoclonal antibodies (Mab's) to the 17 chimeras plus some additional variants of hGH (Table 2). With the possible exception of the pGH(164–190) mutant, disruption of binding to each monoclonal antibody was highly selective and dramatic. For example, the hPRL(88–95), hPRL(97–104), hPL(109–112), and hPRL(111–129) variants did not bind to Mab 1, yet the other mutant hormones outside of these regions bound as efficiently as hGH (Fig. 4). Binding of Mab's 2, 3, 4, 5, and 6 was disrupted by mutations distant in the primary sequence, but in close proximity in the folded hormone. Mab's 1, 7, and 8 were disrupted by mutations defined by a continuous sequence, and one or more of these Mab's may belong to the discontinuous class upon more fine structure mapping.

Table 1. Binding of hGH mutants to the soluble hGH receptor. Some of the mutants were produced by cassette mutagenesis (indicated by cassette), where synthetic DNA duplexes coding for the indicated mutations were ligated into the gap between the closest flanking restriction sites (*21*) in the synthetic gene (*18*). Other mutants were constructed by restriction selection (RS) in which single-stranded synthetic oligonucleotides coded for the desired mutation and inactivated a particular restriction site (*22*). The pGH(164–190) mutant was isolated from a library constructed by random recombination (*14*) of tandemly linked growth hormone genes (hybrid). All sequences were confirmed by dideoxy sequencing (*20*). Cultures of *E. coli* strain W3110 harboring the mutated hGH plasmids were grown in low phosphate media either in 10-liter fermentors or shake flasks (*6*), and the processed hormone that was secreted into the periplasmic space was purified (*41*). All mutant hormones were purified to more than 95 percent homogeneity. Laser densitometric scanning of Coomassie stained gels after SDS-PAGE was used to quantify the concentration of the purified hormones; these values were in close agreement with concentrations determined from the absorbance at 280 nm ($\epsilon_{277}^{0.1\%}$ = 0.92) (*42*). The dissociation constants (K_D) were calculated from Scatchard analysis of competitive binding of ^{125}I-labeled hGH to the cloned and purified soluble hGH receptor at 25°C (*23*). Standard errors in these measurements were generally at or below ±20 percent. Mutants are named on the basis of the first and last residue mutated in the region. The specific mutations introduced are described by a nomenclature where the wild-type hGH residue is given first followed by its sequence position and then the mutant residue. The single letter code designates each amino acid: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. The notation N12H indicates that Asn^{12} has been mutated to His, and i184F indicates a Phe was inserted between residues 184 and 185.

Region probed	Mutant name	Mutations introduced	Mutagenesis method	K_D (n*M*)	$\frac{K_D(\text{mut})}{K_D(\text{wt})}$
	hGH	None		0.34	1.0
A 11 to 33	hPL(12–25)	N12H, F25L	RS	1.4	4.1
	pGH(11–33)	D11A, M14V, H18Q, R19H, F25A, Q29K, E33R	Cassette	1.2	3.4
	hPRL(12–33)	N12R, M14V, L15V, R16L, R19Y, F25S, D26E, Q29S, E30Q, E33K	Cassette	3.6	11
	hPRL(12–19)	N12R, M14V, L15V, R16L, R19Y	RS	5.8	17
	hPRL(22–33)	Q22N, F25S, D26E, Q29S, E30Q, E33K	RS	0.29	0.85
B 46 to 52	hPL(46–52)	Q46H, N47D, P48S, Q49E, L52F	RS	2.5	7.2
	pGH(48–52)	P48A, T50A, S51A, L52F	RS	0.94	2.8
C 54 to 74	hPL(56–64)	E56D, R64M	Cassette	10	30
	pGH(57–73)	S57T, T60A, S62T, N63G, R64K, E65D, T67A, K70R, N72D, L73V	Cassette	5.8	17
	hPRL(54–74)	F54H, S55T, E56S, I58L, P59A, S62E, N63D, R64K, E66Q, T67A, K70M, S71N, N72Q, L73K, E74D	Cassette	23	69
D 88 to 104	hPRL(88–95)	E88G, Q91Y, F92H, R94T, S95E	RS	0.47	1.4
	hPRL(97–104)	F97R, A98G, N99M, S100Q, L101D, V102A, Y103P, G104E	RS	0.53	1.6
E 108 to 136	hPL(109–112)	N109D, V110D, D112H	Cassette	0.61	1.8
	hPRL(111–129)	Y111V, L113I, K115E, D116Q, E118K, E119R, G120L, Q122E, T123G, G126L, R127I, E129S	Cassette	0.52	1.5
	hPRL(126–136)	R127D, L128V, E129H, D130P, G131E, S132T, P133K, R134E, T135N	Cassette	0.58	1.7
F 164 to 190	pGH(164–190)	Y164S, R167K, M170L, D171H, V173A, F176Y, I179V, V180M, Q181K, S184R, i184F, G187S, G190A	Hybrid	≥34	≥100
	pGH(167–181)	R167K, D171H, I179V, Q181K	RS	9.2	27

The set of mutations that disrupted binding to a given Mab could be narrowed either by dissection of a nonbinding chimeric hormone into subdomains (as described for the receptor above) or by eliminating mutations in nonbinding chimeric hormones that were in common with variants that still bound to the Mab. For example, pGH(11–33) retained tight binding to Mab 4, yet hPRL(12–33) disrupted binding. Thus, the disruptive mutations in the hPRL(12–33) variant could be confined to residues not mutated in pGH(11–33)—namely, N12, L15, R16, D26, and E30. This set could be further restricted to N12, L15, and R16 because the hPRL(12–19) disrupted binding, but the hPRL(22–33) did not (Fig. 4). In fact, the N12H mutation in hPL(12–25) could entirely account for the disruption in binding to Mab 4 because this was the only mutation not in common with pGH(11–33). We further tested this hypothesis by mutating Asn^{12} to Ala. Indeed, binding of Mab 4 to the N12A mutant was reduced (100 times less binding), whereas binding to the other Mab's (except Mab 3) was unaffected (Table 2). Thus, it was possible to resolve the epitopes for all eight Mab's even though some of the epitopes partially overlap.

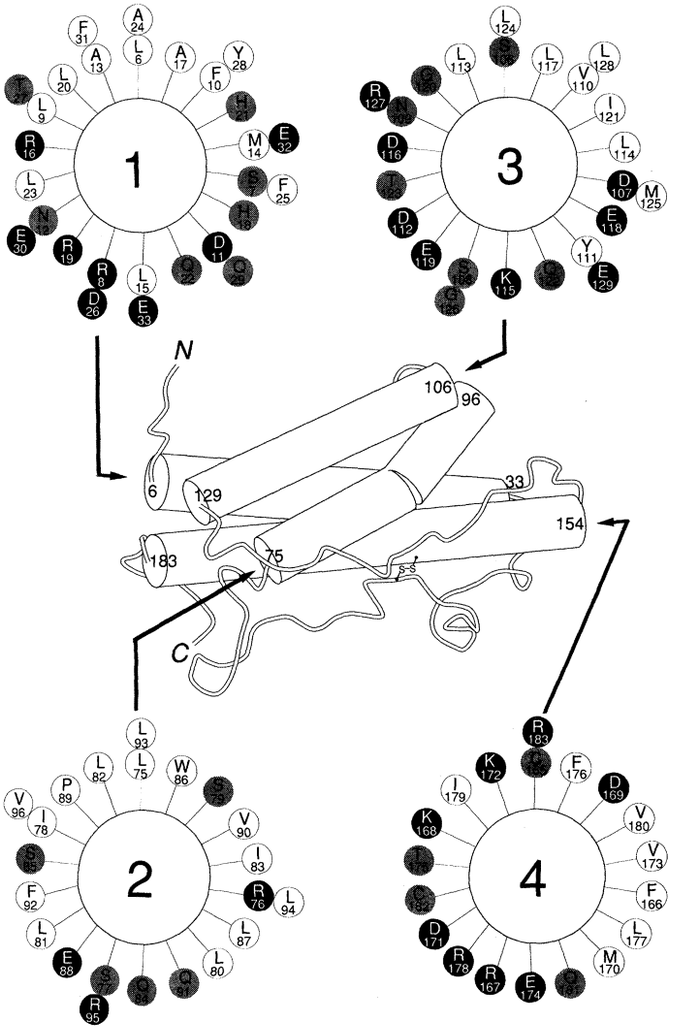

Fig. 2. Structural model of hGH based on a polypeptide chain tracing of the pGH structure (7). The helical start and end points are numbered according to the hGH sequence. Helical wheel projections (*39*) are viewed from the NH_2-terminal start residue of each helix. For helix 4, this projection starts midway with the half-cystine at position 165, which is near the beginning of homologous exchange region F (Fig. 1). Hydrophobic, neutral, and charged residues are indicated by open circle, ○; shaded circle, ◍; and filled circle, ●, respectively.

Although the set of eight Mab's covered most of the hormone, there are still regions where these Mab's did not bind. For example, 4 of the 20 variants did not significantly disrupt binding to any of the Mab's tested [hPRL(22–33), pGH(48–52), hPRL(126–136), and pGH(167–181)]. The sequence of these nonblocking sites may be less antigenic in mice; however, it is more likely that we have not yet tested enough Mab's to isolate ones that are specific for these sites.

Our data show two significant differences between the binding sites for the Mab's and the receptor. (i) The patch defined by disruptive mutations is larger for the receptor than for any of the Mab's. This cannot result from vastly differing affinities because all of these Mab's have binding affinities that are comparable to the receptor [K_D ~0.1 to 1 n*M* (*19*)], except for Mab 1 (K_D ~100 n*M*). (ii) The receptor appears more tolerant of disruptive substitutions in the hormone than are the Mab's. The maximum reduction in binding to the receptor for any of the mutants is about 100 times, whereas almost every antibody has at least one chimera that causes more than a 1000-fold reduction in binding. For some Mab's, much of the reduction can result from a single substitution such as N12A. X-ray crystallographic studies (*25*) have shown that point mutations in hen egg white lysozyme that cause similarly large reductions in binding are in direct contact with the antibody.

Many of the chimeras that caused disruption of receptor binding also disrupted binding to one or more of the Mab's. We therefore evaluated the ability of each of the eight Mab's (normalized to their relative affinities for hGH) to block the binding of the hGH receptor to hGH (Table 3). Mab's 5 and 6 have antigenic determinants located in the loop from 54 to 74 as well as helix 4 that most closely overlap determinants for the receptor (Fig. 4). Accordingly, these Mab's are the most efficient at blocking binding of the hGH receptor. Mab 2 competes almost as well as Mab's 5 and 6 and it too shares a moderately disruptive hGH variant with the receptor [hPRL(12–19)]. Mab 7 competes relatively strongly with the receptor for hGH (Table 3) and it is disrupted by a mutant hormone

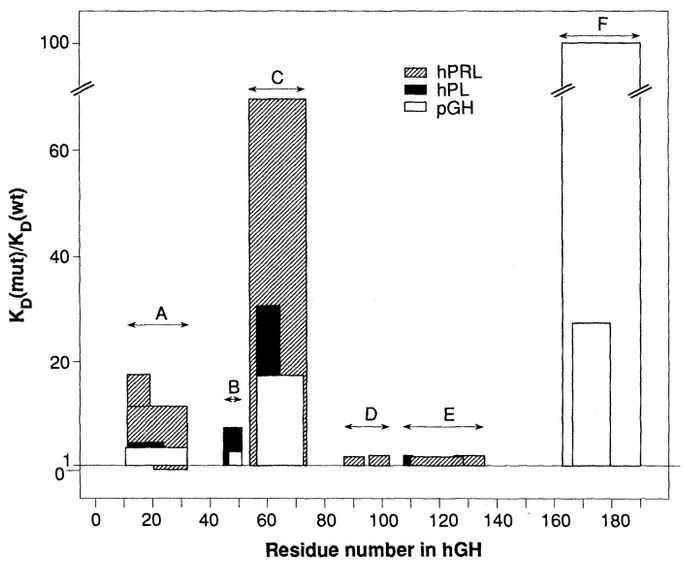

Fig. 3. Bar graph showing the relative reduction in binding to the soluble hGH receptor as a consequence of homolog-scanning mutagenesis of regions A to F. Regions of homologous exchange are shown above each set of bars. The specific mutants within each region are indicated by the shading pattern and the sequence that they span by the bar widths. The reduction in binding is expressed as a ratio of dissociation constants (taken from Table 1). For example, the effect of the hPRL(12–19) mutant on binding is shown by the cross-hatched bar (▨) in region A that extends from residues 12 to 19.

Table 2. Binding of wild-type (wt) and hGH mutants to eight different hGH monoclonal antibodies (Mab's). Rabbit polyclonal antibodies were affinity purified and coated onto microtiter plates (Nunc plates, InterMed, Denmark) at 2 μg/ml (final) in 0.05*M* sodium carbonate (*p*H 10) at 4°C for 16 to 20 hours. Plates were treated with a sample (0.1 μg/ml) of each hGH mutant in buffer B (50 m*M* tris *p*H 7.5, 0.15*M* NaCl, 2 m*M* EDTA, bovine serum albumin at (BSA) 5 mg/ml, 0.05 percent Tween 20 and 0.02 percent sodium azide) for 2 hours at 25°C. Plates were washed and then incubated with the indicated Mab, which was serially diluted from 150 to 0.002 n*M* in buffer B. After 2 hours, plates were washed, stained with horseradish peroxidase conjugated to antibody to mouse immunoglobulin G, and assayed. Values given represent the concentrations (n*M*) of each Mab necessary to produce half-maximal binding to the respective mutant of hGH. Standard errors in these measurements are typically at or below ±30 percent of the reported value. Mab binding data is also shown for a natural variant of hGH (Δ32–46) which deletes residues 32 to 46 in hGH (*12*) and point mutants C182A or N12A.

hGH mutant	Mab (n*M*)*							
	1	2	3	4	5	6	7	8
wt hGH	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
hPL(12–25)	0.4	0.4	>75	>50	0.2	0.2	0.08	0.1
pGH(11–33)	0.4	>100	1.5	0.05	0.2	0.2	0.08	0.1
hPRL(12–33)	0.4	>100	>75	>50	0.2	0.2	0.08	0.1
hPRL(12–19)	0.4	>12	>75	>50	0.2	0.2	0.08	0.1
hPRL(22–33)	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
hPL(46–52)	0.4	0.4	0.1	0.05	0.2	0.2	0.40	0.1
pGH(48–52)	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
hPL(56–64)	0.4	0.4	0.1	0.05	0.2	0.8	0.08	0.1
pGH(57–73)	0.4	0.4	0.1	0.05	>200	>200	0.08	0.1
hPRL(54–74)	0.4	0.4	0.1	0.05	0.2	0.6	0.08	0.1
hPRL(88–95)	>400	0.4	0.1	0.05	0.2	0.2	0.08	0.1
hPRL(97–104)	>400	>12	0.1	0.05	0.2	0.2	0.08	0.1
hPL(109–112)	>12	0.4	>75	15	0.2	0.2	0.08	0.1
hPRL(111–129)	>12	0.4	>75	>50	0.2	0.2	0.08	0.1
hPRL(126–136)	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
pGH(164–190)	0.4	0.4	0.5	0.3	>25	12.5	0.20	0.4
pGH(167–182)	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
hGH(Δ32–46)	0.4	0.4	0.1	0.05	0.2	0.2	>100	>100
N12A	0.4	0.4	>75	>50	0.2	0.2	0.08	0.1
C182A	0.4	0.4	0.1	0.05	2.0	0.2	0.08	0.1

*These Mab's are identified within Genentech as Mab 1, MCA; Mab 2, Hybr 33.2; Mab 3, Medix 1; Mab 4, Hybr 72.3; Mab 5, Medix 2; Mab 6, Chemicon 653; Mab 7, MCD; and Mab 8, MCB.

Table 3. Competitive displacement of hGH receptor from hGH by monoclonal antibodies to hGH. Assays were carried out by immobilization of wild-type hGH in microtiter plates coated with rabbit polyclonal antibodies to hGH as described in Table 2. Receptor (fixed at 10 n*M*) and a given Mab (diluted over a range of 150 to 0.002 n*M*) were added to the hGH-coated microtiter plates for 16 to 20 hours at 25°C, and unbound components were washed away. The amount of bound receptor was quantified by adding a Mab (conjugated to horseradish peroxidase) that bound to the receptor but did not interfere with the binding between hGH and the receptor. The determination of the concentration of each Mab for 50 percent binding to hGH was performed as in Table 2, except that the Mab's were incubated with hGH for 16 to 20 hours instead of 2 hours. The normalized displacement value is calculated from the ratio of the concentration of Mab necessary to displace 50 percent of the receptor to the concentration of Mab necessary to bind 50 percent of the hGH on the plate. This value is used to compare the relative ability of each Mab to displace the receptor.

Mab	Mab (n*M*)*	Mab (n*M*)†	Normalized displacement‡
1	>150	0.4	>375
2	0.8	0.06	13
3	150	0.07	2100
4	150	0.02	7500
5	0.2	0.08	2.5
6	0.2	0.08	2.5
7	0.4	0.04	10
8	>150§	0.04	>3750

*To displace 50 percent of the receptor. †Concentration for binding 50 percent to hGH. ‡Ratios of concentration needed for 50 percent displacement at the concentration needed for 50 percent binding. §Binding of Mab 8 to hGH appears to slightly enhance binding of receptor.

that causes a minor disruption of receptor binding [hPL(46–52)]. Thus, it appears that Mab's 2 and 7 sit on the border of the receptor binding site. Mab's 1 and 8 are unable to give detectable displacement of the receptor, and as expected these contain no overlapping antigenic determinants with the receptor. In contrast, Mab's 3 and 4 are about 1000 times less competitive than Mab 2, yet they also share overlapping disruptive mutations with the receptor and Mab 2 in helix 1 (Fig. 4). This apparent discrepancy may be easily reconciled if the mutations in helix 1 that disrupt Mab's 3 and 4 differ from those residues that disrupt binding to Mab 2 or the receptor. In fact, one such mutant (N12A) disrupts binding of either Mab 3 or 4 without affecting binding to Mab 2 (Table 2) or the receptor (*19*). Thus, it is likely that Mab's 3 and 4 bind to the face of helix 1 (containing N12) that is opposite from the receptor and Mab 2. These competitive binding data taken together with the direct epitope mapping and receptor binding data support the proposed location of the receptor binding site (Fig. 4).

Potential uses and limitations of homolog-scanning mutagenesis. By comparison to antibody competition, homolog-scanning mutagenesis provides a higher resolution map of epitopes in proteins. Antibody competition is of lower resolution because for two Mab's to compete it is only necessary that their epitopes be close or partially overlap, but not superimpose. For example, epitope mapping by competitive binding of 27 different Mab's to hGH resolved a total of only four different antigenic sites on the hormone (*26*). Our data show that the binding sites on hGH for all eight Mab's and the receptor are different although they sometimes overlap. Furthermore, the competitive binding strategy does not locate the epitopes on the sequence. A higher resolution strategy for defining antigenic sites is to test binding to short linear peptides (*27*), or expressed gene fragments (*28*), from the protein of interest. However, for these approaches to work requires that the linear sequence be capable of adopting the conformation found in the intact antigen so that it may be recognized by the Mab. For antibodies to native globular proteins, strictly linear epitopes are predicted on the basis of modeling studies to be rare (*29*).

Similar efforts have been made to map the location of the liver receptor binding site in hGH with peptide fragments (*3, 13, 30*). Collectively, these studies show that no peptide longer than 30 residues retains detectable binding to the liver receptor, and even the large NH_2-terminal fragment (residues 1 to 134) is 50 times lower in binding affinity (*30*). These results can now be rationalized by the model in Fig. 4 because our data show the receptor binding site consists of three discontinuous segments, one of which includes the central portion of helix 4 to the COOH-terminus. This is further supported by the fact that acetylation of Lys^{168} or Lys^{172} in hGH causes a large reduction in receptor binding (*31*). Together, these studies emphasize the importance of mapping discontinuous binding sites in proteins by analysis of structural derivatives of the entire protein.

Several aspects of the homolog-scanning mutagenesis strategy may limit its general utility for mapping epitopes and binding sites in proteins. For a given protein there must exist one or more homologs that do not exhibit the binding property of interest. Such a situation exists for many lymphokine and hormone families including α-interferon, γ-interferon, tumor neurosis factor (TNF), transforming growth factor (TGF-β), epidermal growth factor (EGF), and insulin-like growth factor–1 (IGF-1) as well as their

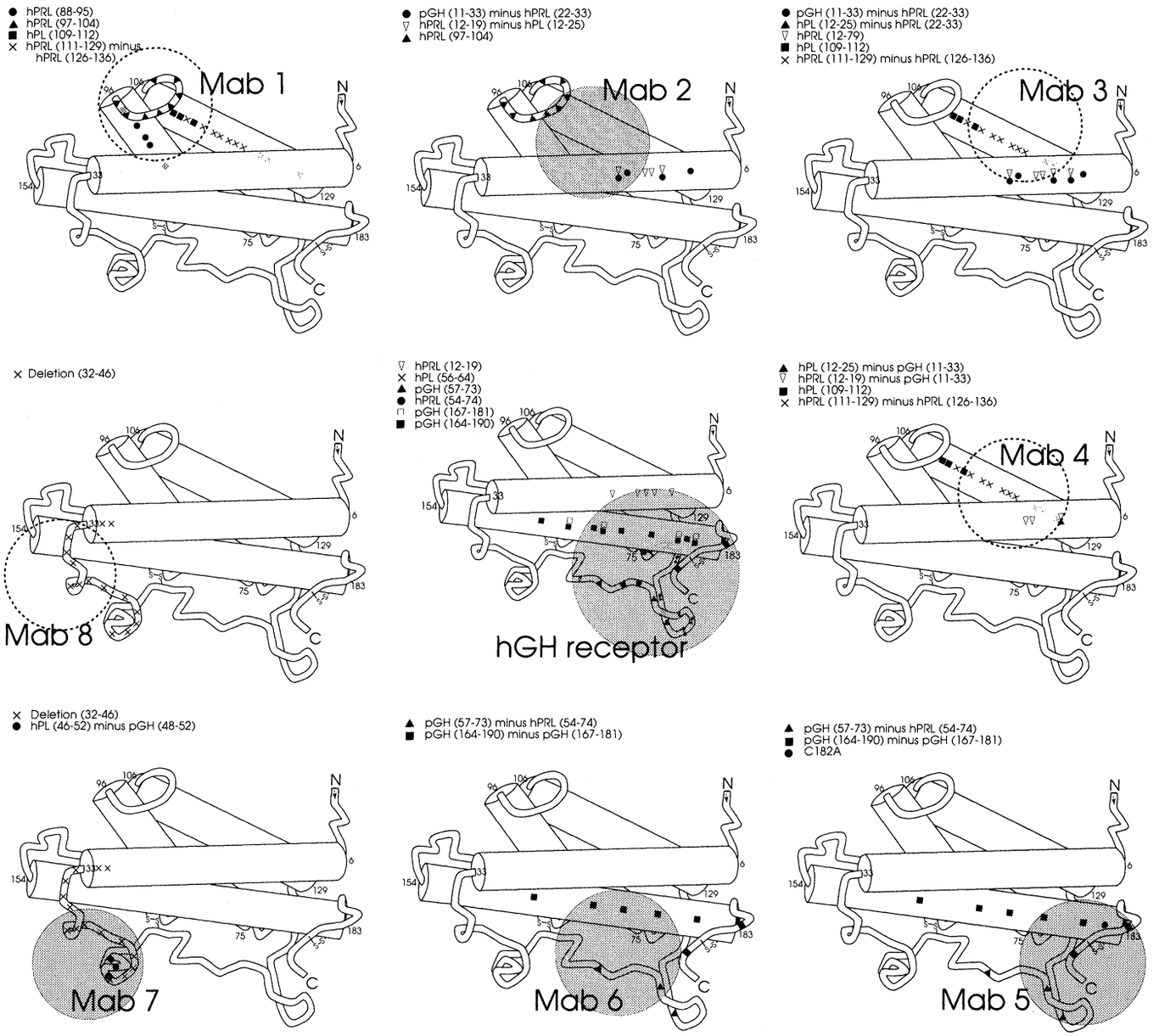

Fig. 4. Location of mutations in hGH (Tables 1 and 2) that disrupt binding by more than a factor of 10 to the extracellular domain of the hGH liver receptor (center) or Mab's 1 to 8 (outside panels arranged in a clockwise fashion). The locations of specific mutations within each chimera are shown by a common symbol. The shaded circles for the Mab's and receptor illustrate the approximate extent and location of the respective contact regions (as defined by the disruptive mutations). The Mab's that block receptor binding are shaded the same as the receptor. This view of hGH was constructed by tracing the back side of the hormone from Fig. 2. The locations of residues along the polypeptide chain were based on information on the known structure of pGH, which is 70 percent identical to hGH and should have virtually the same chain folding as hGH (*8*). Residues in helices were fixed by the interpolating between the indicated start and end points in the projection. Residues in the loop regions were clearly marked by kinks in the pGH structure (*7*). Three small sections of the loops that were not visible in the front view (34 to 36, 97 to 101, 188 to 189) were drawn so as to provide the most direct connection to the next visible structure. The structure from 96 to 106 has been independently determined by nuclear magnetic resonance spectroscopy to be an omega loop structure (*40*).

receptors. It is also desirable to have at least low-resolution structural information so as to avoid destabilizing mutations in buried positions. In the absence of such a structure it may be best to mutate only hydrophilic residues, as was recently done for the interleukin-2 receptor (*32*). In contrast, numerous hybrid proteins have been successfully produced that ignore these structural contraints, including α-amylases (*14*), interferons (*33*), and recently CD4 (*34*) and adrenergic receptors (*35*).

It is possible in any mutagenesis experiment that binding can be disrupted because of a global effect on the protein conformation instead of a direct effect on the binding interaction. There are numerous lines of evidence that indicate the homologous substitutions described here do not disrupt the overall folding of the molecule. Receptor and Mab binding is sensitive to the tertiary as well as primary structure of hGH. The direct mapping data indicate that the receptor and at least five of the Mab's recognize discontinuous epitopes. Furthermore, in control experiments (*36*), binding of an isolated fragment of hGH containing residues 1 to 141 with any of the Mab's (except Mab 8) and the receptor is at least 40 times lower, even though all of the sequence determinants are present in the 1 to 141 fragment for five of these binding proteins (Mab's 1 to 4 and 7). Despite this, each hGH mutant is highly selective in disrupting binding to only one or a small subset of these binding proteins. In one case, the pHG(167–181) mutant causes a 27-fold reduction in receptor binding yet does not affect the binding to any of the eight Mab's. Even the least selective mutant, pGH(164–190), retains tight binding to four of the Mab's. Paterson and co-workers (*37*) have concluded that monoclonal antibodies can be more sensitive probes of conformational integrity than CD (circular dichroism) spectroscopy. Nonetheless, the far ultraviolet circular dichroic spectra of all of the purified hormones tested so far are virtually identical to wild-type hGH (*36*). Finally, all the mutants, with the exception of the pGH(164–190), are expressed in essentially wild-type amounts. Resistance to proteolysis in vivo (during expression) has been used as a screen for conformational integrity (*9*). High resolution structural analysis is still necessary, but together our data indicate that the mutants retain the overall folding pattern of wild-type hGH.

Although mutations do not disrupt the overall folding of the molecule, the fact they disrupt binding does not necessarily implicate those residues as making direct contacts. A disruptive mutation may not only remove a favorable interaction but may introduce an unfavorable one. For example, the N12R mutation in the hPRL(12–19) chimera not only changes the hydrogen bonding amide function of Asn^{12}, but the Arg substitution also introduces a bulkier side chain that is positively charged. Furthermore, a number of the binding contacts may be conserved between the homologs so that not all contacts, or even regions, may be necessarily probed by homolog-scanning mutagenesis. In this regard, a two-chain hybrid containing residues 1 to 134 of hGH and 141 to 191 of hPL (reconstituted from reduced and carboxymethylated peptide fragments) bound as well to rabbit liver membranes as did its homologous hGH derivative (*38*). However, the reverse hybrid was greatly reduced in binding. These data suggested that the receptor contact differences between hPL and hGH were in the 1 to 134 fragment; however, the analysis was insufficient to conclude that all the binding determinants were located in the NH_2-terminal fragment. This is because the 141 to 191 fragment of hPL retains the helix 4 receptor binding determinants, and is nearly identical to hGH in this region (Fig. 1).

The homolog-scanning technique generates a systematic set of multiply mutated proteins that are practical to analyze by quantitative binding analysis. Further fine structural mapping should pinpoint the precise side chains that modulate binding.

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18. The gene was assembled by ligation of seven synthetic DNA cassettes (roughly 60 base pairs (bp) each and sharing a 10-bp overlap with neighboring cassettes) to produce the 405-bp DNA fragment between Nsi I and Bgl II restriction sites (*6*). The ligated fragment was purified from a polyacrylamide gel and cloned into a similarly cut recipient vector, pB0475 (*19*), which contained the alkaline phosphatase promoter and St II signal sequence (*6*), the origin of replication for the phage f1, the pBR322 plasmid origin of replication and the β-lactamase gene from bp 1205 through 4361. The hGH gene sequence was confirmed by dideoxy sequence analysis (*20*). Restriction sites in the synthetic hGH gene began with the following codons: −2, Nsi I; +7, Sal I; +16, Apa I; +22, Nhe I; +33, Stu I; +45, Pst I; +56, Cla I; +65 Nru I; +74, Sac I; +78, Hind III; +87 Xho I; +92, Mst II; +104, Nar I; +107, Asu II; +120, Bam HI; +133, Sac II; +137, Bgl II; +177, Mst I; and +187, Pvu II.
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24. The reduction in binding free energy

$$(\Delta\Delta G_{binding} = +RT \ln [\frac{K_{D(mut)}}{K_{D(wt)}}])$$

is 2 kcal/mol and ~3 kcal/mol for the pGH(167–181) and pGH(164–190) mutants, respectively.
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