antibodies to YTS 169.4 and YTS 191.1, G. Butcher for antibody to P 7/7, P. Crocker for antibodies to M1/70 and SER-4, and M. Shepard for the murine rIFN-γ. Supported in part by Naval Medical Research and Development Command, work unit 3M162770A870AN121 (S.L.H., G.L., A.S., and M.S.), by U.S. Agency for International Development contract DTE0453C003051-00 (M.R.H. and L.W.), and by Office of Naval Research contract N00014-83-C-0355.

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High-Resolution Epitope Mapping of hGH-Receptor Interactions by Alanine-Scanning Mutagenesis

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A strategy, called alanine-scanning mutagenesis, was used to identify specific side chains in human growth hormone (hGH) that strongly modulate binding to the hGH receptor cloned from human liver. Single alanine mutations (62 in total) were introduced at every residue contained within the three discontinuous segments of hGH (residues 2 to 19, 54 to 74, and 167 to 191) that have been implicated in receptor recognition. The alanine scan revealed a cluster of a dozen large side chains that when mutated to alanine each showed more than a four times lower binding affinity to the hGH receptor. Many of these residues that promote binding to the hGH receptor are altered in homologs of hGH (such as placental lactogens and prolactins) that do not bind tightly to the hGH receptor. The overall folding of these mutant proteins was indistinguishable from that of the wild-type hGH, as determined by strong cross-reactivities with seven different conformationally sensitive monoclonal antibodies. The alanine scan also identified at least one side chain, Glu^{174}, that hindered binding because when it was mutated to alanine the receptor affinity increased by more than a factor of four.

SYSTEMATIC REPLACEMENT OF SEGments (7 to 30 residues in length) of human growth hormone (hGH) with sequences derived from nonbinding growth hormone homologs (homolog-scanning mutagenesis) defined a binding patch on a structural model of hGH that included the NH_2-terminal portion of helix 1, a loop between residues 54 and 74 and the COOH-terminal portion of helix 4 (*1*). This analysis provided a general outline of the receptor binding site, but did not identify the specific residues involved in receptor binding. Here, side chains that are important for modulating binding are located by sequential replacement of residues encompassed in the binding patch with alanine. Alanine was chosen as the replacement residue because it eliminates the side chain beyond the β carbon yet does not alter the main-chain conformation (as can glycine or proline) nor does it impose extreme electrostatic or steric effects. Furthermore, alanine is the most abundant amino acid and is found frequently in both buried and exposed positions and all variety of secondary structures (*2*). Alanine-scanning mutagenesis generates a small and systematic set of mutant proteins that can be readily assayed by quantitative binding analysis and avoids the necessity of sorting a library of random mutants by a genetic screen or selection.

A total of 62 single alanine mutants were produced (Table 1) by restriction-selection (*3*) to efficiently enrich for the mutant sequence after primer-directed mutagenesis on a synthetic hGH gene template (*1*). The mutant hormones were expressed in a secreted form from *Escherichia coli* (*4*), and their binding constants were determined for the extracellular portion of the cloned hGH liver receptor by Scatchard analysis (*1, 5*). This receptor fragment is highly soluble and retains high affinity and specificity for hGH (*5*). The use of the purified truncated receptor in binding assays avoided artifacts associated with binding to receptors on membranes or whole cells.

Overall, the results of the alanine scan (Fig. 1) are consistent with those from the homolog scan (*1*) in showing that the middle and COOH-terminal segments are more important in binding than the NH_2-terminal segment. The largest reductions in binding (~20 times lower) occurred for specific alanine substitutions within the 54 to 74 loop and the COOH-terminal sequence 167 to 191. We extended the alanine scan to include residues 2 to 19 because of uncertainties in the positions of the NH_2-terminal residues in the porcine GH (pGH) structure (*6*). In this segment, alanine substitutions caused more modest reductions in binding; the largest reduction (~6 times lower) was for F10A. For one mutant (E174A) that is located near a number of disruptive alanine mutations (Fig. 1), the affinity for the receptor was actually increased (4.5 times higher).

The most disruptive alanine substitutions form a patch that extends from F10 to R64 and from D171 to V185 (Fig. 2). These side chains appear to be facing in the same direction in the model of hGH. For example, all of the alanine mutants tested in helix 4 that most affect binding (D171A, K172A, E174A, F176A, I179A, C182A, and R183A) are confined to three and one-half turns of this helix, and their side chains

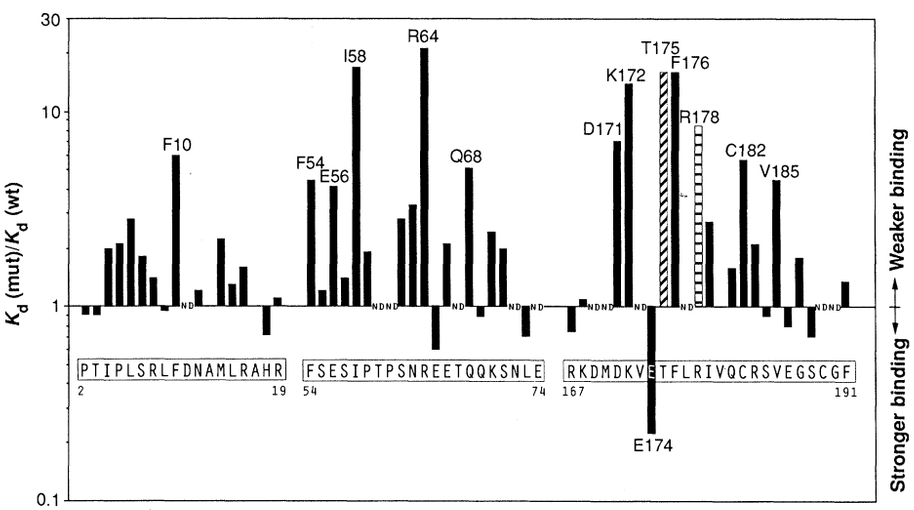

Fig. 1. The change in the dissociation constant relative to wild-type hGH K_d (mut)/K_d (wt) for mutating residues to alanine (black bars), serine (crosshatched bar), or asparagine (hatched bar) within the three binding site segments (Table 1). ND indicates that the K_d (mut) was not determined because of poor expression of the corresponding alanine mutant protein. Residues in which mutations cause a fourfold or greater change in the dissociation constant are labeled. The values are all at or below ±25% (SD).

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project from the same face of the helix (Fig. 2). From this model, it would appear that T175 is involved in binding because it occupies a central position. Although the T175A mutant could not be expressed in high enough yields from cultures grown in shake flasks to be assayed, a more conservative mutant (T175S) could be. Indeed, the T175S mutant caused a 16-fold reduction in affinity for the hGH receptor (Fig. 1). Similarly, although R178A was poorly expressed, a more conservative mutant (R178N) could be expressed and exhibited an eightfold reduction in receptor binding affinity. The next most disruptive mutant in the COOH-terminus was V185A. Although V185A is outside of helix 4, it is predicted by the model to face in the same direction as the disruptive mutations within helix 4. By comparison, alanine mutations tested outside the binding patch (R167A, K168A, E186A, S188A, and F191A), or within it but facing in the opposite direction from those above (V180A, Q181A, and S184A) generally had little or no effect on receptor binding.

A similar interpretation can be made for alanine mutants in helix 1, although the reductions in binding affinity are more moderate. Within the helix, the alanine substitutions that most disrupt binding are at residues 6, 10, and 14, which are located on the same face of the helix. The least disruptive alanine mutations (L9A, N12A, and L15A) are located on the opposite face of helix 1. This is further confirmed by the fact that monoclonal antibodies (Mab's) 3 and 4, which do not compete with the receptor for binding to hGH (*1*), are strongly disrupted by the N12A mutation (Table 2).

The relative positions of side chains within the 54 to 74 loop cannot be fixed from the model as they can be for those within helices 1 and 4. However, there is a striking periodicity in the binding data in which mutations of even numbered residues cause large reductions in binding relative to odd numbered residues. This is especially true for the first part of this region (54 to 59) and may reflect a structure in which even numbered residues project toward the receptor and odd ones away.

Although it is possible that some or all of the alanine mutations that disrupt the receptor binding do so by causing the molecule to be globally misfolded, it is unlikely for the following reasons. (i) Eight different Mab's to hGH (anti-hGH) whose epitopes are distinct from each other (*1*) and seven of which (Mab's 2 to 8) are sensitive to the folded structure of hGH (*7, 8*) bind as tightly with almost all of the alanine mutants that disrupt binding to the receptor as they do with hGH (Table 2). The exceptions are R64A and C182A, which selectively disrupt binding to the Mab's 6 and 5, respectively. It is likely that these mutations disrupt binding determinants in common between the receptor and Mab's 5 and 6 because these Mab's compete with the receptor for binding to hGH (*1*). As additional controls, two alanine mutants are shown that do not affect receptor binding; one of these (N12A) affects the binding of two Mab's and the other (K168A) affects none of the Mab's. Together these data suggest that binding to either the Mab's or receptor is disrupted by a very local perturbation in the mutant structure, the most obvious being the side chain replacement. (ii) The far ultraviolet circular dichroic spectra for seven of these mutants that have been analyzed so far are virtually identical to that of wild-type hGH (*9*). These observations are consistent with crystallographic data showing that single amino acid substitutions in proteins generally cause only small and local structural perturbations apart from the altered side chain (*10*). We cannot exclude the possibility that small perturbations in structure, which are propagated beyond the side chain substitution (*11*), may affect receptor binding (*12*). Thus, the alanine scan identifies side chains in hGH that can most affect binding; such residues are likely but not necessarily in direct contact with the receptor.

About 20% of the alanine mutants (D11A, T60A, P61A, T67A, N72A, E74A, D169A, M170A, V173A, T175A, L177A, R178A, C189A, and G190A) were not secreted at high enough concentrations in shake flask cultures to be isolated and analyzed (Table 1). Each mutant gene was expressed in the same vector and expression was independent of the specific alanine co-

Table 1. Dissociation constants measured for alanine substitutions of residues 2 to 19 in hGH. Site-specific mutagenesis was carried out on a single-stranded template (pB0475) that had unique restriction sites distributed uniformly about every 15 codons throughout a synthetic hGH gene (*1*). Synthetic oligonucleotides (generally 20 to 40 bases long) that coded for the desired alanine (or other) substitution and altered the closest singly occurring restriction site, were used to prime heteroduplex synthesis (*3*). Mutations between codons 2 to 12, 13 to 19, 54 to 60, 61 to 69, or 70 to 74 were generated by primers that hybridized over and altered the restriction sites Sal I, Apa I, Cla I, Nru I, or Sac I starting at codons 7, 16, 56, 65, or 74, respectively. For mutations between codons 167 to 191, we first introduced a Kpn I site at codon 179 that was subsequently altered by restriction selection (*3*). Heteroduplexes were transformed into *E. coli* BMH 71-18 *mutL* (*23*) and the mixture was grown in LB broth (Luria broth) plus ampicillin (50 μg/ml). The mutant sequence was enriched from a mini-lysate of DNA (*24*) by restriction with the enzyme in which the restriction site was altered by the oligonucleotide (*3*). Residual undigested DNA was transformed directly into *E. coli* JM101, and the correct mutants were identified by dideoxy sequencing (*25*). The combined mutagenesis efficiency for recovery of the 64 correct mutants (from ~150 total clones analyzed was about 60%). Mutant and wild-type GH were secreted from *E. coli* W3110 grown in 20 ml of minimal media containing low phosphate for 24 hours at 37°C in 250-ml shake flasks; periplasmic extracts were prepared by osmotic shock (*4*). The growth hormones were purified uniformly to about 60% homogeneity after an $(NH_4)_2SO_4$ precipitation (final concentration equal to 45% saturation) and their concentrations were determined to precision of SD ± 5% by densitometric scanning of Coomassie-stained SDS gels (*1*). Control experiments showed that the contaminating proteins from *E. coli* extracts had no effect on binding of wild-type hGH to the hGH receptor. The dissociation constant for the soluble portion of the cloned liver hGH receptor was determined by competitive displacement of ^{125}I-labeled hGH and Scatchard analysis (*5*). The standard deviations for K_d were at or generally below ±25% of the values reported. Mutants are abbreviated by the wild-type residue (single letter amino acid designation) followed by its codon number and the mutant residue (typically alanine). 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. NE (not expressed) indicates the mutant protein was expressed at below 2% of wild-type hGH as determined by SDS–polyacrylamide gel electrophoresis or enzyme-linked immunosorbent assay (ELISA) after partial purification from *E. coli* periplasmic extracts.

Mutant	K_d (n*M*)	Mutant	K_d (n*M*)
wt	0.34	Q69A	0.31
P2A	0.31	K70A	0.82
T3A	0.31	S71A	0.68
I4A	0.68	N72A	NE
P5A	0.71	L73A	0.24
L6A	0.95	E74A	NE
S7A	0.61		
R8A	0.48	R167A	0.26
L9A	0.32	K168A	0.37
F10A	2.0	D169A	NE
D11A	NE	M170A	NE
N12A	0.40	D171A	2.4
A13(wt)		K172A	4.6
M14A	0.75	V173A	NE
L15A	0.44	E174A	0.075
R16A	0.51	T175A	NE
A17(wt)		T175S	5.9
H18A	0.24	F176A	5.4
R19A	0.37	L177A	NE
		R178A	NE
F54A	1.5	R178N	2.9
S55A	0.41	I179A	0.92
E56A	1.4	V180A	0.34
S57A	0.48	Q181A	0.54
I58A	5.6	C182A	1.9
P59A	0.65	R183A	0.71
T60A	NE	S184A	0.31
P61A	NE	V185A	1.5
S62A	0.95	E186A	0.27
N63A	1.12	G187A	0.61
R64A	7.11	S188A	0.24
E65A	0.20	C189A	NE
E66A	0.71	G190A	NE
T67A	NE	F191A	0.47
Q68A	1.8		

don. We therefore believe variations in steady-state expression reflect differences in the amounts of mutant protein secreted or proteolytic degradation (or both) of the secreted mutant protein. Mutations in buried positions are often destabilizing (*13*) and can lead to proteolysis in vivo (*14*). Indeed, several of the nonexpressing alanine mutants in helix 4 are located on its hydrophobic face (M170A, V173A, and L177A) (Fig. 2). It is likely that mutation of these residues contributed to the instability of an hGH-pGH hybrid that contained residues 1 to 163 of hGH followed by 164 to 191 of pGH (*1*). However, this is not a general effect because several alanine substitutions were tolerated on the hydrophobic face of helix 1 (L6A, L9A, and F10A) and helix 4 (F176A and V180A). In addition to mutations in hydrophobic residues, sometimes replacing charged or neutral side chains with alanine-impaired expression (D11A, T60A, T67A, N72A, E74A, D169A, T175, and R178A). Disruption of the disulfide loop by C182A or C189A mutations leads to very low expression. Several nonexpressing alanine mutants (T60A, P61A, and T67A) were also located in a loop structure containing residues 60 to 67. Thus, although the phenotype of low or nonexpression is characteristic of the mutant protein, this can result from a multitude of structural effects. A more complete interpretation of these results must await a high-resolution structure of hGH and detailed analysis of the rate of secretion and degradation of these molecules in vivo. Moreover, because we have not tested these poorly expressed alanine mutants we do not know whether these wild-type residues are also important for receptor binding.

In two cases tried (T175S and R178N), more conservative substitutions that preserved the hydrogen bonding functional group could be expressed at concentrations near wild type. In another case (C182A), it was possible to purify small amounts of the poorly expressed protein from a high cell density fermentor. Neither of these approaches are very practical for overcoming the problems with poor expression for analysis of large numbers of mutant proteins. However, these alternatives can be applied to a limited extent.

Human growth hormone is a member of a family of hormones that includes the growth hormones (GH), placental lactogens (PL), and the prolactins (PRL) (*15*). Unlike most of its homologs, hGH is capable of binding to somatogenic and lactogenic receptors from a wide range of species. From the alanine-scanning mutagenesis experiments, it is now possible to identify some of the sequence changes that impair binding of hPL, pGH, and hPRL to the soluble hGH receptor (Fig. 3). For hPL, the only differences from hGH among residues where alanine mutations cause more than a fourfold reduction in binding are E56D and R64M. Indeed, when these two hPL residues were introduced into hGH they caused a 30-fold reduction in binding to the hGH receptor (*1*). The combination of these mutations plus I4V and I179M (Table 1) can account (*16*) for greater than a 100-fold reduction in binding for hPL to the somatogenic receptor (*17*). Larger sequence changes occur throughout the three discontinous segments encompassing the binding site for pGH and hPRL (Fig. 3). Of the 13 major sites that alanine replacements cause disruption in binding, 3 are changed in pGH and 9 are changed in hPRL. The combination of these changes can account for a large portion of the reduction in binding for pGH and hPRL to the hGH receptor.

Analysis of sequence divergence among the GH-PRL family has been largely inconclusive in identifying the somatogenic receptor binding site (*15*). The basis for this becomes more clear with information gained from the mutagenesis experiments about the receptor binding site. First, the overall sequence identity for hPL, pGH, and hPRL compared to hGH (85, 68, and 23%, respectively) is essentially the same as the identity within the three discontinuous binding regions (84, 58, and 30%, respectively) (Fig. 3) or within the set of 13 residues where mutations are most disruptive to binding (85, 77 and 31%, respectively). In general, functional differences among these molecules is modulated by sequence differences spread throughout the three discontinuous regions of the binding site. Thus, natural variation within the binding site is no more or less than it is over the entire molecule. Although sequence divergence is a good predictor of the variant binding site regions in such rapidly evolving molecules as protease inhibitors (*18*), it is a poor predictor for identifying sequences responsible for functional differences among the growth hormone family.

Alanine-scanning mutagenesis is a more thorough probe of the receptor binding site than is homolog-scanning mutagenesis (*1*). The latter approach entails anlaysis of multiply mutated proteins, whereas by single substitutions the alanine scan reveals which of these are most crucial in binding (Fig. 2). Moreover the alanine scan identifies important side chains that were missed by the homolog scan such as F10, Q68, and K172. The homolog scan usually introduces conservative side chain substitutions and so may not truly test energetic importance as the alanine scan does. For example, converting Arg 64 to Lys (as in pGH and hPRL) does not reduce binding to the receptor, yet

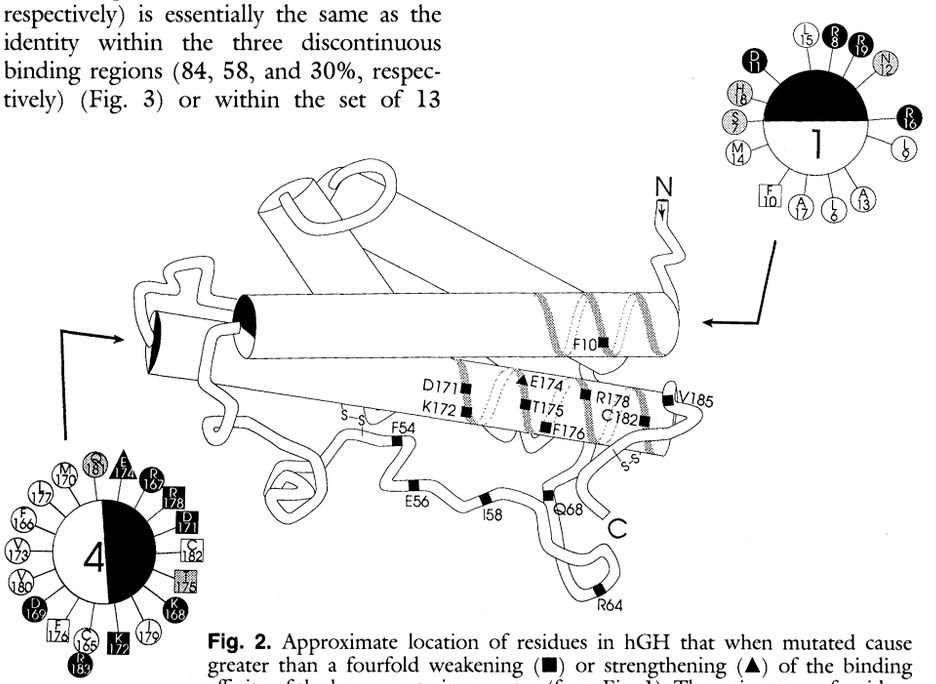

Fig. 2. Approximate location of residues in hGH that when mutated cause greater than a fourfold weakening (■) or strengthening (▲) of the binding affinity of the hormone to its receptor (from Fig. 1). The assignment of residues within each helix was determined by interpolating between the helical boundaries (*6*), and those residues within the loops from the positions of the kinks in the pGH model relative to the disulfides (C53 to C165 and C182 to C189). Helical wheels for helices 1 and 4 are viewed from their corresponding NH_2-terminus (*26*) and include only those helical residues that were mutated (6 to 19 and 165 to 183). These helices are strongly amphipathic (*1*), as shown by the distribution of charged (filled symbols), neutral (shaded symbols), and hydrophobic residues (open symbols). The most hydrophobic side of each helix (nonshaded) is oriented on the model to be facing toward the interior of the protein, which fixes the position of helical residues that most affect receptor binding to be facing outward.

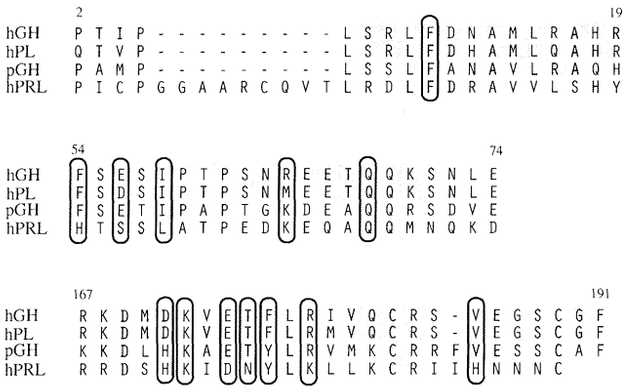

Fig. 3. Comparison of sequences for various members of the GH-PRL family (*15*) within the three segments that strongly modulate binding to the hGH receptor. The shaded residues are identical to hGH and the circled ones are residues that when mutated to alanine (or others) cause the binding constant to change by more than fourfold.

Table 2. Binding of seven different Mab's to hGH and various alanine mutants. An ELISA format was used to determine the concentration (n*M*) of a given Mab necessary to reach 50% saturation in binding to wild-type hGH or various mutants that had been immobilized previously in a microtiter plate (*1*). The standard deviations in these measurements are generally at or below ±30% of the reported value.

Hormone	Mab (n*M*)							
	1	2	3	4	5	6	7	8
hGH	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
F10A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
N12A	0.4	0.4	>75	>50	0.2	0.2	0.08	0.1
I58A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
R64A	0.4	0.4	0.1	0.05	0.2	1.6	0.08	0.1
Q68A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
K168A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
D171A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
K172A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
E174A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
F176A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
C182A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1
V185A	0.4	0.4	0.1	0.05	0.2	0.2	0.08	0.1

conversion of Arg^{64} to Met (as in hPL) reduces binding to the extent of the R64A mutation (*8*). This probably accounts for why the reductions in binding affinity produced by homolog scanning were generally much less than the product of the reductions in binding from component alanine substitutions at the same sites (*16*). For instance, in the mutant pGH (57 to 73) there are ten substitutions but only a 17-fold reduction in binding (*1*). The product of reductions for alanine substitutions at eight of these sites is 273-fold (two of the alanine substitutions were not analyzed because of low expression); most of this discrepancy (~20 times) can be accounted for by the lack of reduction in binding from the R64K substitution as discussed above.

We view the homolog-scanning and alanine-scanning strategies as complementary approaches. The former provides a general location for receptor or antibody epitopes, whereas the latter gives information about specific side chains within a given epitope that are most important for molecular recognition. Both approaches produce a manageable set of mutant molecules that can be analyzed quantitatively. In some instances the mutants could not be expressed in good yield. This problem could sometimes be overcome by selecting more conservative substitutions, or culturing in high cell density fermentors (*1*). In addition, when alanine and glycine side chains are present in the wild-type protein they cannot be probed by mutagenesis except with larger or more conformationally disruptive substitutions, nor can main chain contacts be probed except by potentially disruptive proline substitutions. However, structural analysis of protein-protein interactions have shown that antigenic determinants (*19*) or subunit-subunit interfaces (*20*) are dominated by side chain contacts. In fact, we find the alanine substitutions that most alter binding affinity are replacements of large side chains (both charged and hydrophobic). Finally, disruption of binding by these alanine mutations does not prove a residue is making contact, further structural analysis of the complex is necessary for that.

Although it is anticipated that all epitopes in native globular proteins are to some extent discontinuous (*21*), they invariably contain multiple contacts within a short continuous stretch (<15 residues) of the polypeptide chain (*19*). Thus, once a putative contact region is identified by mutagenesis, chemical modification or another method, the importance of neighboring residues to binding affinity can be assessed by alanine scanning. For example, recently it was reported that alkylation of K172 in hGH substantially reduces binding (*22*). Our data show that other nearby residues in helix 4 are indeed crucial in receptor binding. Finally, alanine scanning has identified at least one side chain in hGH (E174) that naturally disrupts binding to the hGH receptor. Thus, it is likely that the binding between hGH and its somatogenic receptor may be strengthened by optimizing this and other residues that modulate binding. Alanine-scanning mutagenesis is a systematic functional analysis that should be of general use to identify (and subsequently engineer) the side chains that most strongly modulate the interaction between a polypeptide hormone and its receptor (or in other protein-ligand complexes).

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Physical Mapping of a Translocation Breakpoint in Neurofibromatosis

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The gene for von Recklinghausen neurofibromatosis (NF1), one of the most common autosomal-dominant disorders of humans, was recently mapped to chromosome 17 by linkage analysis. The identification of two NF1 patients with balanced translocations that involved chromosome 17q11.2 suggests that the disease can arise by gross rearrangement of the *NF1* locus, and that the NF1 gene might be identified by cloning the region around these translocation breakpoints. To further define the region of these translocations, a series of chromosome 17 Not I–linking clones has been mapped to proximal 17q and studied by pulsed-field gel electrophoresis. One clone, 17L1 (D17S133), clearly identifies the breakpoint in an NF1 patient with a t(1;17) translocation. A 2.3-megabase pulsed-field map of this region was constructed and indicates that the NF1 breakpoint is only 10 to 240 kilobases away from 17L1. This finding prepares the way for the cloning of *NF1*.

VON RECKLINGHAUSEN NEUROFIBROMATOSIS (NF1) is an autosomal-dominant human genetic disease, characterized by café-au-lait spots, multiple neurofibromas that increase in size and number with age, hamartomas of the iris (Lisch nodules), learning disabilities, bone abnormalities, and an increased risk of malignancy (especially glioma and neurofibrosarcoma) (*1*). The incidence of the disease is about 1 in 4000. The specific manifestations and severity are remarkably variable, even within the same family, and the spontaneous mutation rate is high, with 30 to 50% of cases representing new mutations (*2*).

The NF1 gene (*NF1*) has been recently mapped to chromosome 17 by linkage analysis (*3*), and genetic analysis of 142 families by an International Consortium has indicated that *NF1* lies on proximal 17q (*4*). Markers on both sides of the gene have been identified that are within 5 centimorgans of *NF1* (*5*).

In support of this localization, two unrelated patients with NF1 and apparently balanced translocations involving chromosome 17 [t(1;17) and t(17;22)] have been identified (*6, 7*). In each instance, the chromosome 17 breakpoint is in band q11.2, precisely where *NF1* maps by linkage analysis. Somatic cell hybrids have been constructed that contain the translocation chromosomes from these patients (*7, 8*). Flanking genetic markers for *NF1* map on opposite sides of the translocation breakpoints (*7–10*), supporting the hypothesis that these translocations directly disrupt the gene. Other somatic cell hybrids created by microcell-mediated gene transfer have been used to further define the location of markers around the *NF1* locus (*9, 10*). The combination of linkage and physical mapping has now ruled out several chromosome 17 genes, including *ERBA1*, *ERBB2*, and *NGFR*, as candidates for direct involvement in NF1 (*9–11*).

Thus *NF1* is an appropriate target for cloning by reverse genetics (*12*). We have reported (*9*) initial results of physical mapping of this region by pulsed-field gel electrophoresis (PFGE). Although these results allowed us to physically connect some of the closely linked genetic markers, gaps were present in the map and none of these markers detected either NF1 translocation breakpoint. Therefore additional markers were required to visualize this region in more detail. The use of linking clones, which are genomic fragments containing rare restriction sites (*13*), has advantages in such an effort: such clones allow convenient construction of a physical map, and often mark the site of expressed genes. We generated a phage library of Not I–linking clones from flow-sorted chromosome 17 material (*14*) and localized these clones using a somatic cell hybrid panel (*9*). The clones that mapped to 17q11 were tested on PFGE blots (*15*) to see whether abnormal fragments were present in DNA from either NF1 patient with a translocation.

Of 16 linking clones studied, one (called 17L1) identified novel PFGE bands in DNA from the t(1;17) NF1 patient. Specifically, a 0.8-kb Not I–Xho I fragment of 17L1, denoted 17L1A, detected abnormal fragments with the enzymes Bss HII, Sac II, and Not I (Fig. 1A) and also with Mlu I. No abnormality was seen with Sfi I, Eag I, or Xho I (*16*).

A potential pitfall in this analysis can arise from variability in cutting of rare restriction sites. This can occur as a result of actual sequence polymorphism (*17*) or, more commonly, as a result of DNA methylation differences (*15*). Strong evidence that such effects cannot account for the data shown here include: (i) No novel PFGE fragments have been seen with 17L1A in Not I analysis of more than 60 normal chromosomes in several different tissues. (ii) These fragments cannot be accounted for by the effects of incomplete digestion; for example, the intentional partial Not I digest in Fig. 1A demonstrates that the t(1;17)-specific band at 550 kb (lane 9) is distinct from the 460- and 680-kb partial fragments in normal individuals (lanes 10 and 11). (iii) The t(1;17) DNA, which also includes a normal chromosome 17, always shows a normal band as well as the abnormal band, in approximately a 1:1 ratio. (iv) The abnor-

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