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A Single Glutamic Acid Residue Plays a Key Role in the Transcriptional Activation Function of Lambda Repressor

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Summary

Previous experiments have suggested that negative charge is an important aspect of the activating region of lambda repressor as it is for at least one class of eukaryotic transcriptional activators. Here we randomize amino acids in the activating region of repressor and assay the function of over 100 variants. We find that acidic residues at the four solvent-exposed positions on the surface of an α helix (helix 2 in the structure) together comprise a strong activating region. Only one of these acidic residues, however, is critical for activation, and at this position glutamate is strongly preferred to aspartate. At the three remaining positions, certain uncharged residues (different ones at each position) function as well as or better than the acidic residues. Basic residues, however, are highly detrimental to function at all four positions. Our mutagenesis studies also suggest limitations on amino acid substitutions that allow formation of the helix-turn-helix DNA binding motif found in repressor and in many other DNA binding regulatory proteins.

Introduction

The phage lambda repressor activates transcription when bound adjacent to P_{RM}, the promoter for its own gene (for review and references, see Ptashne, 1986). According to our present understanding, repressor bound to the operator site O_R2 increases the rate of initiation of transcription at P_{RM} by touching RNA polymerase. The "activating region" of repressor, that which evidently touches polymerase, is an acidic patch on repressor's surface. Evidence for this view came in part from the analysis of mutants that bind to lambda operators but stimulate transcription weakly if at all. These "positive control" (pc) mutants cluster in a patch on repressor's surface that lies close to RNA polymerase when polymerase is bound at P_{RM} and repressor is bound at O_R2. Each of the substitutions in the positive control mutants makes the activating region less acidic. A pseudorevertant of one of the positive control mutants, conversely, restores activity by substituting an acidic residue for an uncharged residue in the activating region (Guarente et al., 1982; Hochschild et al., 1983).

The activating region and the DNA binding region of repressor lie in the same domain. This part of repressor contains a "helix-turn-helix" motif, composed of helices 2 and 3 in the structure, that mediates specific binding to lambda operators. In the repressor–operator complex, helix 3 is inserted into the operator's major groove, allowing amino acids on the surface of the helix to interact with the base pair edges, and helix 2 lies across the major groove and anchors helix 3 in place. The positive control mutants alter residues in helix 2 and in the turn between helices 2 and 3 that are solvent-exposed in the repressor–operator complex (Jordan and Pabo, 1988).

The idea that repressor contains a discrete surface for activation is reinforced by the finding that the activating region can function in the context of another protein. We converted lambda Cro—a helix-turn-helix containing protein that normally represses transcription—into an activator by replacing residues in the helix-turn-helix of Cro with those required for activation in repressor. The modified Cro bound at O_R2 activates transcription, whereas wild-type Cro bound at this site does not (Bushman and Ptashne, 1988).

The nature of the activating region of lambda repressor is of particular interest in view of the finding that acidic regions also comprise activating regions in eukaryotes. Many eukaryotic activating regions, for example those of the yeast proteins GAL4 and GCN4 and the herpes virus protein VP16, are characterized by a net negative charge (Hope and Struhl, 1986; Ma and Ptashne, 1987a; Triezenberg et al., 1988). Mutants of a GAL4 derivative that improved activation usually made the activating region more acidic (Gill and Ptashne, 1987), and new activating regions encoded by random fragments of E. coli DNA were found to have a net negative charge (Ma and Ptashne, 1987b). Certain eukaryotic acidic activators have been shown to function in a wide array of eukaryotes (Fischer et al., 1988; Kakidani and Ptashne, 1988; Lin et al., 1988; Ma et al., 1988; Webster et al., 1988).

In this study, we used DNA synthesis to replace the part of the repressor gene (*cI*) encoding its activating region with random DNA. We isolated a wide array of mutant derivatives, all of which encode proteins that retain the ability to bind to lambda operators, and measured their abilities to activate transcription at P_{RM}. Analysis of these mutants shows that there are four solvent-exposed positions on helix 2 where the presence of acidic amino acids favors activation. However, at only one of these positions is an acidic residue crucial, and we describe efficient activators bearing neutral residues at the remaining three positions. At these positions basic residues are most unfavorable for activation. We also describe a series of variants bearing alterations at other positions in helix 2 and in the turn between helices 2 and 3.

Results

Replacing Parts of *cI* with Random DNA

The part of *cI* encompassing the sites of the original positive control mutants was mutagenized by replacing parts

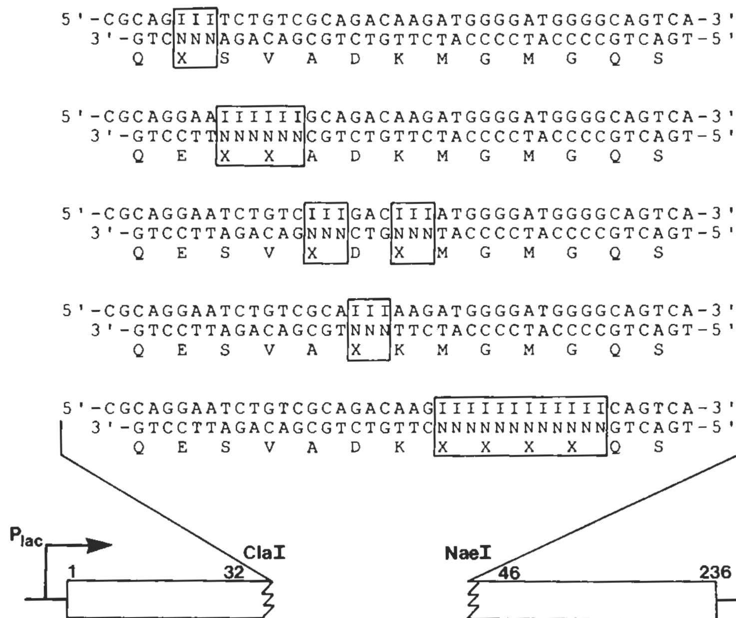

Figure 1. Schematic Illustration of Our Mutagenesis Procedure

cI, the gene for repressor (shown as an open rectangle), was mutagenized by replacing parts of it with synthetic oligonucleotides (shown above *cI*). *cI* transcription is controlled by P_{lac}. The ClaI and NaeI sites used in our constructions are as indicated. The amino acids encoded by each oligonucleotide are shown beneath the DNA sequence. X = all amino acids. At positions in the DNA marked as N, a mixture of A, G, C, and T was used during oligonucleotide synthesis. At positions indicated as I, inosine was used. Codons mutagenized using each oligonucleotide are boxed. The numbers refer to the position in the repressor amino acid sequence. Positive control mutants were isolated by Guarente et al. (1982) and Hochschild et al. (1983) at positions 34, 38, and 43. The single letter amino acid code is 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, Y = Tyr.

of it with random DNA (see Figure 1). As a vector for this manipulation, we used a fusion of P_{lac} to *cI* carried on a multicopy plasmid. This plasmid directs the synthesis of lambda repressor as roughly 1% of cellular protein (500 times more than a normal lysogen) (Levine et al., 1979). Restriction sites were introduced into *cI* bracketing the DNA encoding helix 2 and the turn between helices 2 and 3 (amino acid residues 34–43). Complementary pairs of oligonucleotides were then used to replace the DNA between these restriction sites. Each pair of oligonucleotides restored the wild-type coding sequence except at chosen codons which were replaced with random DNA. Each randomized codon contained a mixture of all four bases at each of the three positions on one strand, and inosine at the corresponding positions on the other strand. Inosine can pair with each of the four bases in DNA and therefore facilitates hybridization of the DNA strands (Reidhaar-Olson and Sauer, 1988). Each pair of oligonucleotides was separately ligated with vector DNA to make a "library" of *cI* derivatives containing all possible codons at predetermined positions.

Repressor mutants that retain the ability to bind to lambda operators were identified by introducing each library into E. coli by transformation, and then plating cells onto plates seeded with lambda phages mutant in *cI*. The only cells that survive are those that synthesize repressor derivatives that can bind to lambda operators and block phage growth. Each derivative of repressor that passed our selection was tested for its ability to stimulate transcription from P_{RM} in vivo. Plasmid encoded mutants were introduced into a bacterial strain containing a P_{RM}–*lacZ* fusion in place of the endogenous *lac* operon, and the activity of P_{RM} was monitored by assaying β-galactosidase. Our procedure, it should be noted, is designed to measure the activation function of repressor molecules bound to O_R2, and not to reflect possible differences in the affinities of various mutants for DNA (see Experimental Procedures, Interpreting the Assay).

Figure 2 presents the sequences of our mutants and their phenotypes. The sequence of wild-type repressor is shown at the top; residues contained in helix 2 (33–39) are enclosed in the cylinder, and residues in the turn between helices 2 and 3 (40–43) are shown in extended form. The substitutions found in repressor derivatives from cells that survived our selection are shown beneath the wild-type sequence in columns. Acidic residues are enclosed in dark ovals and basic residues in open ovals. The efficiency of transcriptional activation, measured as units of β-galactosidase from a P_{RM}–*lac Z* fusion, is indicated to the right of each column. Note that different numbers of codons were mutagenized in different experiments. Positions 34 and 38 were each mutagenized alone, so there is only one amino acid in the column associated with each of these positions. Positions 35 and 36, and 37 and 39, were mutagenized together using a pair of oligonucleotides that replaced both codons with random DNA, and the columns in Figure 2 describing these experiments therefore show two amino acids in each line. The four positions in the turn were all mutagenized together, and hence the corresponding column has four amino acids in each line.

The derivatives of repressor are ranked in Figure 2 according to the efficiency with which they activate. Stimulation above the basal level of 63 units ranges from nil to 17-fold. Wild-type stimulates transcription about 7-fold (469 units), and so our collection of mutants contains both impaired and improved activators.

Figure 2 reveals that four positions on the solvent-exposed surface of helix 2 can be altered to either improve or impair activation (here and below "solvent-exposed"

Gln 33 · Glu 34 · Ser 35 · Val 36 — Ala 37 · Asp 38 · Lys 39 · Met 40 · Gly 41 · Met 42 · Gly 43

34	
Glu	469
Asp	147
Ser	94
Ile	94
Tyr	84
Leu	74
Pro	71
Cys	69
Gly	64
Val	62
Ala	60
Thr	56
His	55
Asn	53
Lys	43

35	36	
Leu	Val	1060
Asp	Val	1056
Asp	Thr	999
Val	Val	971
Glu	Ala	881
Thr	Val	865
Ile	Leu	820
Leu	Met	800
Ile	Ala	778
Gln	Ala	679
Val	Leu	636
Leu	Thr	591
Ser	Thr	585
Ala	Ala	562
Thr	Ala	554
Ala	Leu	550
Ile	Ile	508
Gly	Leu	503
Ala	Thr	489
Ser	Val	469
Gly	Ala	465
Gly	Cys	456
His	Leu	443
Thr	Ile	391
Tyr	Ala	343
Tyr	Ile	322
Gly	Ile	317
Arg	Val	238
Arg	Ile	188
Lys	Ser	68

37	38	39	
Ser	Asp	Glu	1029
Ala	-	Asn	870
Ser	-	Val	868
Ala	-	Ile	867
Ala	-	Gly	836
Ala	-	Leu	832
Ser	-	Leu	829
Ser	-	Cys	800
Ala	-	Phe	794
Ser	-	Gly	767
Gly	-	Ile	730
Gly	-	Gly	715
Gly	-	Tyr	711
Gly	-	Met	691
Gly	-	Ala	677
Ser	-	Tyr	669
Gly	-	Val	622
Gly	-	Leu	619
Ala	-	Arg	572
Ser	-	Lys	537
Ala	-	Lys	469

38	
Tyr	614
Asp	469
Ala	255
Glu	218
Leu	201
Gly	187
Asn	101
Pro	93
Val	88
Gln	81
Lys	52
Arg	51

40	41	42	43	
Ile	Asn	Met	Ser	677
Leu	Leu	Met	Gly	675
Ile	Ser	Met	Gly	656
Met	Asp	Arg	Ser	591
Leu	Ile	Met	Gly	561
Leu	Leu	Ile	Ser	541
Met	Ser	Val	Gly	536
Met	Gly	Met	Gly	469
Leu	Thr	Ile	Gly	464
Met	Ser	Val	Gly	408
Leu	Gly	Lys	Gly	399
Ala	Lys	Met	Gly	390
Ile	Ser	Leu	Ser	357
Val	Gly	Tyr	Gly	306
Val	Gly	Arg	Ser	298
Leu	Arg	Ile	Gly	271
Val	Gly	Arg	Thr	265
Val	Gly	Ile	Tyr	219
Leu	Tyr	Lys	Ser	218
Met	Glu	Lys	Cys	217
Val	Arg	Lys	Ser	176
Met	Met	Phe	Gly	171
Met	Arg	Lys	Cys	132
Leu	Tyr	Arg	Ser	100
Leu	Arg	Tyr	Cys	97
Met	Ser	Val	Arg	70
Val	Lys	Ile	Arg	65
Ile	Gly	Tyr	Arg	63
Met	Ser	Phe	Arg	63
Met	Ser	Ile	Lys	61
Leu	Arg	Met	Arg	61
Met	Asn	Ile	Arg	61
Met	Tyr	Ile	Arg	60
Ile	Gly	Arg	Lys	59
Met	Arg	Phe	Arg	52

Figure 2. Sequences and Stimulating Activities of Wild-Type and Mutant Derivatives of Lambda Repressor

The sequence of wild-type lambda repressor is shown at the top of the figure. Residues 33–39 (helix 2) are shown as an α helix; residues 40–43 (the turn) are shown in extended form. Amino acid substitutions isolated at each position are shown as columns joined to the wild-type residues by lines. The wild-type sequence is enclosed in a rectangle in each column. Acidic residues are enclosed in dark ovals, basic residues are enclosed in open ovals. The numbers beside each column indicated the units of β-galactosidase measured in cells containing a plasmid directing the synthesis of the indicated mutant protein; each cell also contained a fusion of P_{RM} to *lacZ* to measure activation. The basal level of transcription from P_{RM} (i.e., the level in the absence of repressor) is 69 units. The wild-type protein gives 469 units.

refers to the dispositon of these positions in the repressor–operator complex). Each of these positions is highlighted in red in the space-filling model of the repressor–operator complex shown in Figure 3. In general, the presence of acidic residues at these positions correlates with efficient activation and the presence of basic residues correlates with poor activation. Most uncharged residues impair activation, but at three of the solvent-exposed positions, an uncharged residue can function as well as or better than the acidic residues. In the next two sections we will consider each of the positions in helix 2 and in the turn.

Helix 2

Position 34

The residue at this position lies on the solvent-exposed surface of helix 2 (Pabo and Lewis, 1982; Jordan and Pabo, 1988). Fifteen different amino acids can be accommodated here (i.e., we found 15 variants at this position among proteins that confer immunity in our selection). However, only proteins containing Glu (the wild-type residue) at this position activate transcription substantially. A mutant containing Asp at this position activates transcription only 2-fold above the basal level, and mutants containing uncharged residues activate to less than 50% above the basal level.

Position 35

The residue at this position is also solvent-exposed, and 14 different amino acids can be accommodated. Proteins containing acidic side chains at this position—either Glu or Asp—are among the best activators, but so are proteins containing Leu and Val. Most proteins containing uncharged side chains at this position are less effective activators than those containing acidic side chains, and proteins containing basic side chains activate least well.

Position 36

The residue at this position forms part of the hydrophobic core, and eight different (mostly hydrophobic) residues are found here among our mutants. The variants show slight differences in their abilities to activate; for example,

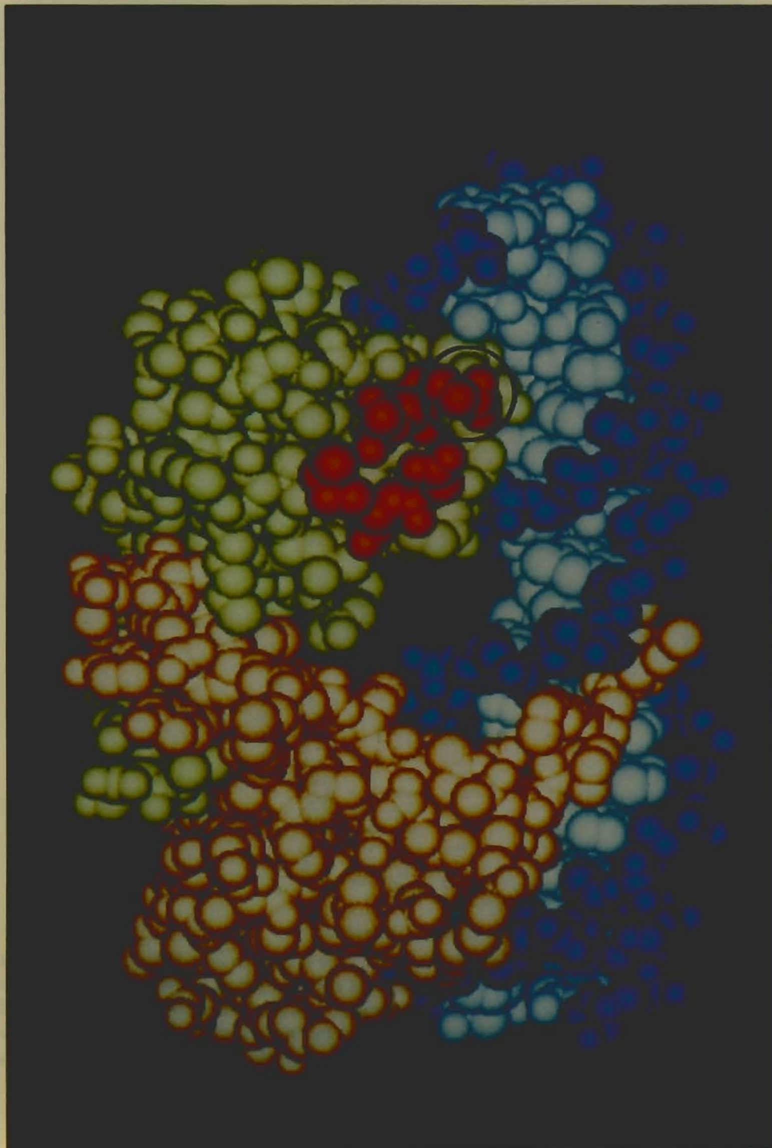

Figure 3. Space-Filling Model of a Complex between the Amino-Terminal DNA Binding Domain of Lambda Repressor and a Lambda Operator Site, with the Four Positions Important for Activation Highlighted

The DNA is colored blue and white, the two monomers of repressor are colored yellow and orange. The four residues on the surface of helix 2 that are important for activation (positions 34, 35, 38, and 39) are colored red, and Glu 34 is circled. The structure of the complex shown in the figure was determined by Jordan and Pabo (1988).

the protein containing Leu 35 and Val 36 activates transcription 2-fold better than a protein containing Leu 35 and Thr 36.

Position 37

The residue at this position is packed against the polypeptide backbone of helix 3. Only the three shortest amino acid side chains—Gly, Ala, and Ser—can be accommodated at this position, and proteins containing these substitutions activate about equally well.

Position 38

The residue at this position is solvent-exposed and repressor can accommodate 12 different amino acids here. A protein containing Tyr at 38 activates most effectively, followed by a protein containing the wild-type Asp. A protein with Glu at this position activates transcription 2-fold less well than wild-type, as do proteins containing the shortened side chains of Ala or Gly. Proteins containing other uncharged or basic side chains at 38 are further impaired for activation.

Position 39

The residue at this position is also solvent-exposed and 14 different amino acid residues can be accommodated. A protein containing Glu activates most effectively, proteins containing uncharged residues activate somewhat less effectively, and proteins containing basic residues, including the wild-type Lys, activate least effectively.

The Turn

All of the residues in the turn were replaced simultaneously, so each amino acid substitution was isolated in the context of several other changes. Certain regularities are discernable, however, and these are discussed below.

Position 40

The residue at this position, the most amino-terminal in the turn, comprises part of the hydrophobic core of the protein. Only five different residues, all hydrophobic, can be accommodated here. The different allowable residues have no obvious effect on activation.

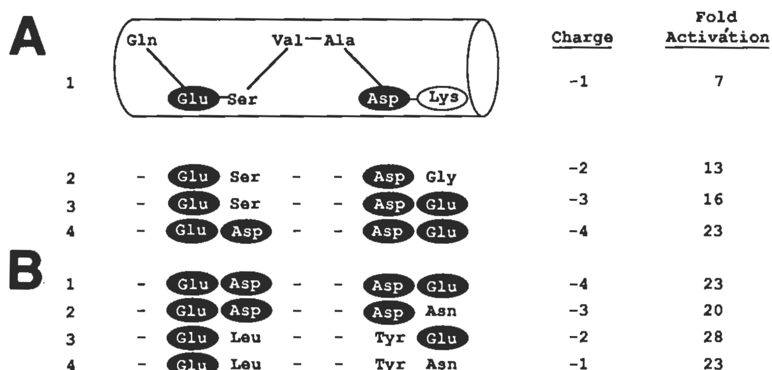

Figure 4. Selected Mutants That Illustrate the Two Ways of Making a Strong Activating Region

The wild-type amino acid sequence is shown at the top, with the residues in helix 2 enclosed in a cylinder. Acidic residues are enclosed in dark ovals, and basic residues are enclosed in open ovals. (A) Increasing negative charge increases the activity of the activating region (lines 1–4). (B) Replacing negatively charged residues with chosen uncharged residues also suffices to make strong activating regions (lines 1–4).

Position 41

The residue at this position is solvent-exposed, and 11 different amino acids can be accommodated here. Proteins containing basic amino acids at this position activate transcription poorly. Proteins containing the other allowable substitutions here show no striking differences in activation.

Position 42

The residue at this position is part of the hydrophobic core. Eight different amino acid residues can be accommodated at this position, all of which have hydrophobic character. Perhaps Lys and Arg, which are among the residues allowed at this position, adjust their conformations so that the aliphatic methylene groups lie in the protein core and the positively charged groups protrude into solvent. Proteins containing basic residues at this position activate poorly; proteins containing other substitutions activate about equally well.

Position 43

The residue at this position is solvent-exposed, in the structure, and 7 different amino acids can be accommodated. None of these seven residues are hydrophobic, perhaps because the residue at 43 interacts with DNA when repressor binds to operator. The most effective activators have Gly or Ser at this position. Repressor derivatives containing large amino acid side chains or basic side chains at this position activate poorly.

Constructing Very Strong Activators

Three very strong activators were generated by combining residues found to favor activation in the experiment in Figure 2. First, we constructed a derivative containing only acidic amino acids on the solvent-exposed surface of helix 2. This protein stimulated transcription 23-fold, compared with 7-fold for wild-type (Figure 4A, lines 1 and 4). Next, we constructed a mutant that contains the most effective residues for activation on the solvent-exposed surface of helix 2 as found in the experiments of Figure 2. That is, at each of the four solvent-exposed positions, we inserted the residue found at the top of the appropriate column of Figure 2. This strategy yielded our strongest activator, which stimulated transcription 28-fold (Figure 4B, line 3). A similar mutant containing optimal residues both on the solvent-exposed surface of helix 2 and in the turn activated transcription equally well (data not shown).

Glu 34 Is Critical for Activation in Very Strong Activators

Our strongest activator, (see Figure 4B, line 3) contains only two acidic residues in the activating region, Glu 34 and Glu 39. To examine the relative importance of these residues, each was separately replaced with an uncharged residue. Replacing Glu 34 with Ser nearly abolished activation (Figure 5, line 1). In contrast, replacing Glu 39 with Asn had little effect (Figure 5, line 2). Similar phenotypes

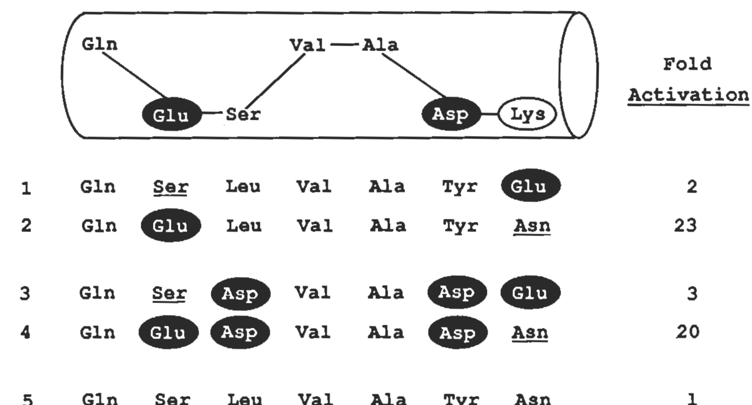

Figure 5. The Roles of Glu 34 and Glu 39

Glu34 and Glu 39 were each replaced separately with an uncharged residue in two of our strongest activators. The activator with the optimal residues on the surface of helix 2 (Figure 4B, line 3) was altered to contain Ser at 34 (line 1) or Asn at 39 (line 2). The activator containing four acidic residues on the surface of helix 2 (Figure 4A, line 4) was altered to contain Ser at 34 (line 3) or Asn at 39 (line 4). An activator with optimal uncharged residues at positions 34–43 (and so containing Ser 34 and Asn 39) is presented in line 5. The sequence of this protein at positions 40–43 is Met Ser Gln Ser.

were obtained by making the same replacements in the context of our most acidic activator (Figure 5, lines 3 and 4). Substituting Gln for Glu 34 in the strong activator of Figure 4B, line 4, abolished activity (Figure 5, line 5). Thus Glu 34 is critical for activation even in the context of our strongest activators.

Discussion

Elements of the Transcriptional Activating Region of Lambda Repressor

Our results suggest that at only one position on the surface of repressor—position (34), the second from the amino terminus of α helix 2—is an acidic residue required for the transcriptional activation function. Even in the context of other residues highly favorable for activation, substitution of Glu 34 with an uncharged residue drastically decreases activity and, moreover, glutamate functions significantly better than aspartate. In contrast, at the three remaining solvent-exposed positions on helix 2, any of several uncharged residues function as well as acidic residues, whereas in every case mutants bearing basic residues at any of these positions are least active.

Figure 4 presents selections from our data that illustrate some of these points. Figure 4A presents a set of activators that increase in strength with increasing acidity at the four solvent-exposed positions on helix 2. An activator containing all acidic residues at these positions stimulates transcription 23-fold (line 4). Wild-type repressor, which has a net negative charge of −1 at these positions, stimulates transcription 7-fold (line 1). Two proteins with net negative charges of −2 and −3 at these positions have intermediate phenotypes (lines 2 and 3). Figure 4B presents a contrasting set of activators that have net negative charges ranging from −4 to −1, all of which stimulate transcription 20-fold or more (lines 1–4). Note that the uncharged amino acids in these mutants were chosen because they are found in strong activators in the experiment of Figure 2.

Our results confirm and extend the conclusions drawn from the analysis of the original three positive control mutants (pc 1–3 of Guarente et al., 1982, and Hochschild et al., 1983). In mutant pc3, the critical Glu 34 residue is changed to a Lys, thereby removing the one residue found in this study to be required for efficient activation and replacing it with a particularly unfavorable basic residue. The mutant pc2 bears Asn in place of the solvent-exposed Asp at position 38, thus removing an acidic residue favorable for activation. Asn at position 38 apparently interferes with activation, since substitution at this position with Gly or Ala is less detrimental to the activation function. In pc1, Gly 43, a residue in the turn, is replaced by Arg. In the context of wild-type repressor, alterations in the turn do not greatly improve activation. (The only exception to this rule is a revertant of a positive control mutant that introduced an acidic amino acid into the turn; this change may have been allowable only because the initial positive control mutant made the protein less acidic in this region. See Hochschild et al., 1983). Changes in the turn that introduce basic residues, however, greatly impair activation. Thus mutant pc1 is an example of such a change.

Implicit in our results is the conclusion that wild-type repressor is not optimized for activation. At position 39, for example, the wild-type Lys residue is actually the least effective for activation of any residue tested. It is possible that the activating region of wild-type repressor could not be substantially improved without interfering with binding of repressor at the levels found in a lysogen; we have found that several of our mutants bind DNA less tightly than does wild-type repressor as judged by experiments performed in vivo (data not shown).

What are the functions of the four residues that comprise the activating region? Because of its critical importance for activation, it seems plausible that Glu 34 interacts specifically with RNA polymerase. We do not know of a precedence that might help explain the interchangeability of acidic and certain neutral residues at the remaining three solvent-exposed positions on helix 2. It may be relevant in this regard that among nine point mutants of a eukaryotic activator that increased the activation function, all increased negative charge by replacing a basic residue with a neutral one (Gill and Ptashne, 1987). Perhaps the absence of positively charged residues will prove to be particularly characteristic of activating regions in both prokaryotes and eukaryotes.

Requirements for Binding and Folding

Our results identify several requirements for proper DNA binding and protein folding, some of which may have general implications for identifying helix-turn-helix motifs in amino acid sequences. We find that amino acid side chains extending into the hydrophobic core of the protein (residues 36, 37, 40, and 42) can tolerate fewer substitutions (and allow DNA binding) than the side chains of amino acids that are bathed in solvent (residue 43, an exception to this rule, is discussed below). The restrictions at 37 are most striking: only the three shortest amino acid side chains—Gly, Ala, and Ser—can be tolerated at this position. In the helix-turn-helix containing proteins, this residue is packed against the backbone of helix 3 (Mondragon et al., 1989). Thus, as has been suggested previously (Pabo and Sauer, 1984), a short side chain at this position is probably required for formation of the helix-turn-helix.

The restrictions on allowable residues at 43 probably derives from the proximity of this side chain to DNA in the repressor–operator complex. In wild-type repressor, the main chain NH group of Gly 43 forms a hydrogen bond with a nearby phosphate (Jordan and Pabo, 1988). Mutant derivatives containing polar side chains at 43 may form hydrogen bonds with phosphates, and derivatives containing basic side chains may form salt bridges with the phosphate backbone.

None of our functional turn sequences contain Pro, indicating that the presence of Pro in the turn probably disrupts function. To explore this view, we altered *cI* so as to replace Gly 43 with Pro, and found that this abolished binding of repressor (data not shown). It seems probable that the tight turn between helices 2 and 3 cannot form when Pro, the least flexible of the amino acids, is present in the turn. Alternatively, the Pro side chain may sterically

interfere with DNA binding by colliding either with DNA or side chains of repressor that interact with DNA.

Trp and His are both also absent from our collection of turn sequences. These amino acids probably cannot pack properly into the hydrophobic core of the protein, explaining their absence from positions 40 and 42. Possibly these bulky residues interfere with DNA binding when present at positions 41 and 43. We note, however, that the codons for Trp and His are relatively uncommon, so these residues could be missing from our collection by chance.

Three rules for forming the helix-turn-helix in lambda repressor can be tentatively extended to all proteins containing this structure. First, the third residue from the carboxy terminus of the first helix (residue 37 in lambda repressor) must be Ser, Gly, or Ala (see also Pabo and Sauer, 1984). Second, the last residue in the turn (residue 43 in lambda repressor) must be able to interact with DNA. Suitable residues found in this study include Gly, Ser, Arg, Lys, Cys, Thr, and Tyr. This list may have to be expanded slightly: P22 repressor almost certainly contains a helix-turn-helix (Wharton and Ptashne, 1987), and it has an Asn at this position. Third, proline is apparently not allowable in the turn.

The structures of six helix-turn-helix containing proteins, lambda repressor, lambda Cro, 434 repressor, 434 Cro, CAP, and Trp repressor, have been solved using X-ray diffraction, and these proteins generally conform to the above three rules (Pabo and Lewis, 1982; Anderson et al., 1981; Anderson et al., 1985; Wolberger et al., 1988; McKay and Steitz, 1981; Schevitz et al., 1985). None of the six contain Pro in the turn, and all have residues from the allowable set at the last position in the turn (residue 43 in lambda repressor). Only five of the six proteins, however, have Ser, Ala, or Gly at the third from the last position in the first helix (residue 37 in repressor). Trp repressor has a Lys residue at this position. Lys cannot be accommodated at position 37 in lambda repressor: our genetic screen is saturated at position 37 and only Gly, Ser, and Ala are found. In addition, molecular modeling studies indicate that Lys could not be tolerated at this position by lambda repressor or 434 repressor without a compensating change in the relationship between helices 2 and 3. Perhaps Trp repressor adjusts the structure of its helix-turn-helix to accommodate the Lys residue, making the helix-turn-helix of Trp repressor a member of a slightly different class of structural motifs (A. Aggarwal, personal communication).

Experimental Procedures

Bacterial Strains and Methods

Bacterial strains used in this study are as described in Meyer et al. (1980). The selection for lambda immunity was conducted as described in Backman et al. (1977). β-galactosidase assays were performed as described by Miller (1972). Because strains containing plasmids that direct the expression of lambda repressor derivatives at high levels lose plasmids frequently (unpublished data), the subcultures used for β-galactosidase assays were regularly tested for lambda immunity after the assays were performed. DNA transformations were conducted as described (Maniatis et al., 1982).

Plasmid Constructions

The vector plasmid used to mutagenize *cI* was constructed as follows. First, a DNA fragment containing lambda *cI* under control of P_{lac} was excised from the plasmid pUC19-cI (A. Astromoff, unpublished data) on an EcoRI–ClaI restriction fragment, and ligated to pUC18 (Messing, 1983) digested with the restriction enzymes EcoRI and NarI. This manipulation yielded plasmid pCS1. All ligation reactions and subsequent plasmid isolations were conducted essentially as described in Maniatis et al. (1982). Separately, the plasmid pAH22 (Hochschild and Ptashne, 1986) was sequentially mutagenized using oligonucleotides of sequence 5′-GACAGATTCATCGATAAGCCAAGTT-3′ and 5′-GCACCAACGCCGGCCTGCCCCATC-3′ according to the method of Zoller and Smith (1983), yielding plasmid pCS3. The mismatched bases used to generate mutations in *cI* are underlined. These manipulations introduced a ClaI and a NaeI restriction enzyme recognition site into *cI* in the positions indicated in Figure 1. Next, the EcoRI to HindIII fragment from pCS3 was ligated with pCS1 digested with EcoRI and HindIII. One isolate with the proper structure was named pCC1. Finally, an EcoRI fragment from pFB64 (Bushman and Ptashne, 1988) containing the *lacI* gene was ligated with pCC1 digested with EcoRI. This manipulation, which yielded plasmid pCC2, served to reduce the level of expression of the repressor derivatives under control of P_{lac}. The sequence of one isolate of pCC2 was determined in the region of the promoter and the novel NaeI and ClaI sites, and found to be as expected.

Construction and Analysis of Plasmid Libraries

Libraries containing all possible codons at predetermined positions were constructed essentially as described by Reidhaar-Olson and Sauer (1988). First, the vector pCC2 was digested with ClaI and NaeI. Because it was difficult to cut to completion with NaeI, pCC2 was first digested with NaeI only, linear DNA molecules were gel isolated on 5% acrylamide gels, DNA was recovered by electroelution (Maniatis et al., 1982), and only then were molecules cut with ClaI. Oligonucleotides listed in Figure 1 were treated with kinase and ATP as described (Maniatis et al., 1982) and ligated with the doubly digested pCC2 DNA. After treatment with ligase overnight, each ligation mixture was extracted with phenol, precipitated with ethanol, and cut with ClaI. This manipulation served to inactivate any reclosed vector molecules present in the ligation mix. The sequence of each oligonucleotide was chosen to restore the defects in *cI* created in making the ClaI and NaeI sites; thus the only source of plasmids conferring lambda immunity was vector molecules joined to the desired oligonucleotides.

Plasmids were sequenced using the dideoxy method and double-stranded DNA templates (Sanger et al., 1980). Sequence analysis showed that oligonucleotides synthesized by the Harvard Microchemistry Facility on an Applied Biosystems DNA synthesizer were adequately random. We encountered one unexplained departure from randomness: the Gly codon GGG was greatly overrepresented in the libraries that mutagenized position 34 and positions 37 and 39.

Site-directed mutagenesis of the activating region of repressor was performed by ligating the double stranded oligonucleotides listed in Table 1 into pCC2 digested with ClaI and NaeI. Subsequent work up and analysis of ligation products was as described for our libraries.

Interpreting the Assay

We believe that activation by our mutants cannot be explained by differential occupancy of the O_R2 site. The alterations that improve activation relative to wild-type cannot act by increasing binding to O_R2, because wild-type repressor is present in our experiment at levels more than sufficient to fill O_R2, and thus mutants could not stimulate transcription more by filling O_R2 further. In the case of the mutants impaired for activation, our selection does not guarantee that repressor is bound to O_R2: repressor bound only to O_R1 (an operator site adjacent to O_R2) can be sufficient for immunity (see Ptashne, 1986) and so we cannot be certain that O_R2 is occupied. We know of no example, however, of wild-type repressor or any mutant derivative of the amino terminal domain binding to O_R1 but not O_R2, and in the one case where we isolated a mutant repressor protein (Glu34→Ala) and studied its binding in vitro, O_R1 and O_R2 filled simultaneously (unpublished data).

Differences in activation among our mutants are also unlikely to be due to binding to the third operator site, O_R3, which overlaps P_{RM} (wild-type repressor negatively regulates P_{RM} by binding to O_R3). The P_{RM}–*lac Z* fusion used in this study contained two mutations in O_R3 that drastically reduce binding of repressor (Meyer et al., 1980). Wild-type repressor does not appear to fill O_R3 substantially under the conditions of our experiment, because lowering the amount of repressor

Table 1. Oligonucleotides Used to Direct Changes in *cI*

(1)	5'-CGCAGGAAGACGTCGCAGACGAAATGGGGATGGGGCAGTCA-3'
	3'-GTCCTTCTGCAGCGTCTGCTTTACCCCTACCCCGTCAGT-5'
	Q E D V A D E M G M G Q S
(2)	5'-CGCAGGAACTGGTCGCATATGAAATGGGGATGGGGCAGTCA-3'
	3'-GTCCTTGACCAGCGTATACTTTACCCCTACCCCGTCAGT-5'
	Q E L V A Y E M G M G Q S
(3)	5'-CGCAGTCACTGGTCGCATATAATATCAATATGTCACAGTCA-3'
	3'-GTCAGTGACCAGCGTATATTATAGTTATACAGTGTCAGT-5'
	Q S L V A Y N I N M S Q S
(4)	5'-CGCAGTCACTGGTCGCATATGAAATGGGGATGGGGCAGTCA-3'
	3'-GTCAGTGACCAGCGTATACTTTACCCCTACCCCGTCAGT-5'
	Q S L V A Y E M G M G Q S
(5)	5'-CGCAGGAACTGGTCGCATATAATATGGGGATGGGGCAGTCA-3'
	3'-GTCCTTGACCAGCGTATATTATACCCCTACCCCGTCAGT-5'
	Q E L V A Y N M G M G Q S
(6)	5'-CGCAGTCAGACGTCGCAGACGAAATGGGGATGGGGCAGTCA-3'
	3'-GTCAGTCTGCAGCGTCTGCTTTACCCCTACCCCGTCAGT-5'
	Q S D V A D E M G M G Q S
(7)	5'-CGCAGGAAGACGTCGCAGACAATATGGGGATGGGGCAGTCA-3'
	3'-GTCCTTCTGCAGCGTCTGTTATACCCCTACCCCGTCAGT-5'
	Q E D V A D N M G M G Q S

The pair of oligonucleotides used for each construction is shown. The encoded amino acid sequence is shown beneath the DNA sequence. Departures from the wild-type amino acid sequence are underlined. Each pair of oligonucleotides was used to construct the repressor derivative indicated below by its position in Figure 4 or Figure 5:
(1) → Figure 4A, line 4
(2) → Figure 4B, line 3
(3) → Figure 5, line 5
(4) → Figure 5, line 1
(5) → Figure 5, line 2
(6) → Figure 5, line 3
(7) → Figure 5, line 4

does not significantly increase the activity of P_{RM} (data not shown). It is unlikely that our mutants fill O_R3 more than wild-type does, because 28 out of 30 of our mutants bound DNA less tightly than did wild-type repressor, as assayed in vivo in a test that monitored binding to a single weak operator (data not shown). The two mutants that bound DNA more tightly than wild-type were Glu 34→Lys and Ala 37→Gly, Lys 39→Gly. The former mutant was previously shown to have higher affinity for operator (Nelson and Sauer, 1985).

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