Identification of the DNA binding domain of the phage λ cII transcriptional activator and the direct correlation of cII protein stability with its oligomeric forms

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The bacteriophage λ transcriptional activator protein cII is a DNA-binding protein that coordinately regulates transcription from phage promoters important for lysogenic growth. We have genetically and structurally characterized more than 80 different single amino acid substitutions in this 97-amino-acid protein. A subset of 25 of these variant proteins was utilized for detailed biochemical analysis, which allows us to define specific domains critical for sequence-selective DNA recognition, nonspecific DNA binding, and protein oligomerization. The mutation studies also demonstrated the remarkable correlation of oligomeric structure of cII protein to its stability within the bacterial host. An *Escherichia coli* HtpR⁻ strain has been identified that greatly stabilizes these highly unstable cII mutants.

[Key Words: DNA-binding domain; λ cII; transcriptional activator; protein stability]

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The cII protein of phage λ is a positive regulator of gene transcription that plays a pivotal role in the lytic versus lysogenic development decision made by the phage within infected cells. The cII activator coordinately regulates transcription of three separate transcription units, each controlled by a cII-dependent promoter signal, P_{RE}, P_L, and P_{aQ}, respectively (Fig. 1). Two of the cII-dependent promoters, P_{RE} and P_L, control the expression of the two phage gene products, repressor (cI) and integrase (*int*), respectively, which are required for integration of the phage DNA into the host genome and its maintenance in a dormant state (Herskowitz and Hagen 1980; Wulff and Rosenberg 1983). The third promoter, P_{aQ}, is thought to direct the synthesis of an antisense RNA, the function of which is to reduce the expression of the Q gene product (Ho and Rosenberg 1985; Hoopes and McClure 1985), necessary for the expression of all phage late genes during lytic growth. Because cII binding and transcription activation of P_{RE}, P_L, and P_{aQ} occur at essentially the same cII levels (Ho et al. 1983; Ho and Rosenberg 1985), these three promoters function in concert to achieve the lysogenic growth response of the phage.

cII is a tetrameric protein consisting of four identical subunits, each containing 95 amino acids with a subunit molecular weight of 11,000 daltons (Ho et al. 1983). cII is a DNA-binding protein that binds selectively in the homologous -35 regions of all three promoters. DNA protection experiments and mutational analyses demonstrated that the cII binding site is comprised of tetranu-

cleotide repeat sequence, TTGCN₆TTGC (Ho et al. 1983). In the presence of cII protein, RNA polymerase binds cooperatively to the promoter regions of P_{RE}, P_L, and P_{aQ}. The polymerase contacts occur primarily on the opposite face of the DNA helix from where cII binds and, thus, cII and RNA polymerase appear to sandwich the DNA between them (Ho et al. 1983).

The cII protein is different from several other well-characterized sequence specific DNA-binding proteins in that it recognizes a direct repeat sequence rather than a DNA sequence with twofold rotational symmetry, as is the case with the lambda repressor (cI) protein and Cro protein and the *Escherichia coli* CRP protein, Trp repressor, and Lac repressor (Gilbert and Maxam 1973; Dickson et al. 1975, 1977; Dykes 1975; Maniatis et al. 1975; Gilbert et al. 1976; Bennett et al. 1977; Humayun et al. 1977; Johnson et al. 1978). At present, we do not know whether the cII tetramer has a twofold axis of symmetry or an asymmetric arrangement. If it does have a twofold symmetry like the other DNA-binding proteins noted above, it is unclear how this arrangement will recognize a directly repeated DNA sequence.

Our prior studies have focused primarily on the DNA sequences involved in promoter function and positive regulation by cII. In this paper our attention shifts to the structure and function of the cII regulatory protein itself. We select and characterize more than 80 different point changes in the 97-amino-acid cII-coding region. A subset of these mutations is used to analyze the domain struc-

Figure 1. Schematic diagram showing the coordinately controlled cII-dependent transcription units on phage λ . Thick horizontal arrows indicate origin and direction of transcription from the three cII-activated promoters, P_I , P_{RE} , and P_{aQ} (Ho and Rosenberg 1985). Also indicated is the proposed transcription terminator of the P_{aQ} transcription unit t_{aQ} . Thin arrows indicate the origin and direction transcription from the promoters P_I and P_R , which control the major leftward and rightward operons of phage. The approximate position of the genes encoding integrase (*int*), N protein, repressor (*cI*), cII protein, and Q protein are also shown.

ture of the cII protein and define the relevance of various regions of the protein to cII function. These single amino acid substitutions in cII are used to characterize the ability of the protein to interact with DNA both selectively and nonselectively, to activate transcription, to form tetramers, and to exist stably within the bacterial host cell.

Results and discussion

Single amino acid substitutions in the cII gene

A variety of mutations have been characterized previously that map within the cII gene coding region of phage λ (Brachet and Thomas 1969; Rosenberg et al. 1978; Wulff et al. 1980; Wulff et al. 1984) these mutants were characterized genetically, and representative mutations were selected for DNA sequence analysis. More than 80 different single amino acid substitutions were defined in this analysis, and these are summarized in Table 1 and Figure 2. The cII mutants can be divided functionally into three groups based on phenotype and genetic complementation (Wulff et al. 1984). The largest group represents cII⁻ mutants, which exhibit little or no cII function. A smaller group of mutants, designated cII⁺⁻, exhibit partial cII activity, and the third group, designated cII⁺, are phenotypically similar to the wild-type cII. The variant cII proteins represented by these mutations provide the basis for a detailed analysis of the structure–function relationships between different regions of the cII protein.

In an effort to obtain biochemical data on the various cII mutant classes, a representative set of 25 mutants with single amino acid alterations spanning the entire cII protein were cloned into the *E. coli* expression vector system, pK30, under the transcriptional control of the inducible phage λ P_L promoter. This allowed us to induce the expression of each of these cII derivatives rapidly and efficiently. The following sections describe the genetic, structural, and biochemical studies that were performed on these mutants, allowing us to assign the cII protein into a set of specific structure–function domains (Fig. 3).

Sequence-specific DNA-binding domain

Crystallographic study of several different DNA binding regulatory proteins has indicated that these proteins use a common structural motif to recognize their specific DNA binding sites (Anderson et al. 1981; McKay and Steitz 1981; Olandorf et al. 1982; Pabo and Lewis 1982; Pabo and Sauer 1984). This motif consists of two α -helical regions separated by a short ‘turn’ which permits the two helices to be juxtaposed at the appropriate angle required for their interaction with DNA. This general protein structure is referred to as the helix-turn-helix motif. It has been noted that a variety of other DNA-binding proteins, including λ cII protein, contain regions with some structural homology to the helix-turn-helix motif (Mathews et al. 1982; Pabo and Sauer 1984). In the case of cII, the region of homology extends from amino acid position 26 to 45 (see Fig. 4A).

We have characterized more than 20 different amino acid substitutions in this region of cII (Fig. 4B). Genetic analysis indicates that most of these mutants (those shown below the line in Fig. 4B) are defective in positive regulation. Seventeen different amino acid substitutions were defined in the postulated second α -helical DNA-binding region of the cII protein (Figs. 3 and 4). We have overexpressed and purified five of these mutants: cII 3563 (Lys-37 to Arg), cII SDM-1 (Lys-37 to Glu), cII 3062 (Ser-38 to Leu), cII 3378 (Ser-41 to Gly), and cII 3151 (Arg-42 to Gly). Sedimentation analysis indicated that all five of these mutant proteins retained their ability to form tetramers (see Table 2). Apparently, mutations in this region have little effect on overall cII structure, particularly as it pertains to the ability of the molecule to oligomerize. Another class of mutants defective in tetramer formation that lie outside and flank this region will be discussed in detail below. DNase protection experiments (Fig. 5A) demonstrated that these mutants have lost their ability to bind selectively (i.e., footprint) to the cII recognition domain on DNA, which is in the –35 region of the P_{RE} promoter. Moreover, even at concentrations of protein greater than 10-fold higher than those used for the wild-type protein, these mutants showed no activator activity in *in vitro* transcription reactions (Fig. 5B). Clearly, the mutations in this region

Table 1. Amino acid substitutions in the *cII* gene

Codon	Wild type	Mutant ^{a,b}		Codon	Wild type	Mutant ^{a,b}	
2	val	leu*	ALA*	asp*	33	val	ala
3	arg	CYS ^c			34	gly	ser*
4	ala	THR ^{c*}	VAL ^d		37	lys	arg* glu ^{e*}
5	asn	ASP ^d			38	ser	pro leu*
6	lys	glu	ARG ^d	ASN ^{d,f}	40	ile	asn* thr <u>met</u> leu
7	arg	CYS ^{a,d}	HIS ^d	CYS	41	ser	gly* asn ile
8	asn	ASP ^d	HIS ^d	SER ^d	42	arg	gly* trp* lys met ser
9	glu	gly*	ASP ^c		44	lys	glu
10	ala	thr	val		46	asp	gly* SER ^g
11	leu	pro			47	trp	arg
13	ile	phe			51	phe	tyr ser
14	glu	lys			52	ser	leu pro
15	ser	asn			53	met	LYS*
17	leu	ser			55	leu	phe*
19	asn	asp			58	leu	phe
21	ile	thr	asn		59	glu	lys*
24	leu	pro*			62	val	<u>ala</u>
25	gly	glu			67	met	thr*
26	thr	ile			68	ala	<u>thr</u>
27	glu	LYS*			70	leu	ser* phe* trp
28	lys	glu*			71	ala	thr val
29	thr	<u>ile</u>			74	val	<u>ile</u> ala
30	ala	thr			75	ala	<u>thr</u>
31	glu	LYS*			78	leu	<u>phe</u> pro
32	ala	<u>ser</u> *	<u>val</u>		92	gln	<u>arg</u> *
					95	met	<u>val</u>

Nonsense and frameshift mutations are not shown.

^a Mutant key: aaa = *cII*⁻ phenotype; aaa = *cII* partial activity; AAA = *cII*⁺ phenotype.

^b * indicates these *cII* mutants cloned into the pKC30 expression vector (see text for details).

^c Revertants of Leu-2 or Asp-2.

^d cy P_{RE} mutants (see review by Wulff and Rosenberg 1983).

^e Created by site directed mutagenesis (see Methods).

^f Bracket represents double mutation.

^g A revertant of Gly-46.

c II Mutations

Figure 2. Positions of amino acid substitution mutations in the λ cII gene. The amino acid sequence of λ protein is shown using the standard single-letter code. The solid vertical lines below the sequence indicate amino acid positions of *cII* mutants that are defective in function. The solid and dotted vertical lines above the sequence indicate amino acid positions of mutant that maintain full or partial *cII* function, respectively. The bracket indicates a double mutation.

define a domain of *cII* protein important for DNA interaction (Fig. 3). These results agree with the mutational studies on Trp repressor, that most of the mutations decreasing or eliminating repressor activity affect residues in the proposed second α -helical DNA binding region (Kelley and Yanofsky 1985). The fact that many of these second helix mutations are strikingly conservative amino acid changes (e.g., Lys-37 to Arg, Ser-46 to Gly, Arg-42 to Lys) suggests that the specific side chains of these amino acid residues, rather than their polarities in general, are important for DNA interaction. This biochemical evidence clearly indicates that the second helical region plays a key role in the sequence-specific DNA-protein recognition. This finding is also consistent with the prediction made from the helix-turn-helix model, which suggests that this region penetrates into the major groove of the DNA and makes highly specific contacts with the nucleotide bases exhibited in this groove.

Although these second helix mutations eliminate specific *cII*-DNA interactions, most of these mutants (four of the five tested) retain their ability to bind DNA non-specifically. This was demonstrated using DNA agarose-affinity chromatography, a method commonly used in

No. of amino acid	2	9	26	33	37	46	72	97
	NH ₂		helix		helix		COOH	
No. of mutants	15	14	8	2	17	19	7	
Activation function	+	-	±	-	-	-	±	
Tetramer structure	yes	no	yes	yes	yes	no	yes	

Figure 3. Domain structure of the cII protein (see text for details).

the purification of DNA binding proteins and a method that was used in the purification of wild-type cII (Ho et al. 1982). The second helix variants were able to bind to the DNA matrix and were released when salt concentrations in the elution buffer reached between 0.1 and 0.17 M versus 0.25 M salt for the wild-type protein. There is one mutant in the second helix that exhibits no binding at all (Ser-38 to Leu). Apparently, most of the mutations in the second helix do not affect the helix-turn-helix structure radically and thereby allow some nonspecific DNA interaction to be retained even in the absence of specificity. We presume that the Ser-38 to hydrophobic Leu substitution must alter the helix-turn-helix conformation significantly, thereby resulting in total loss of the nonspecific DNA binding property. Most important, the data indicate that the proposed 9-amino-acid-long second α -helical region in cII is the only region of the protein in which substitutions result in the selective loss of sequence-specific DNA binding while main-

taining the ability to form tetramers and bind DNA non-specifically. These properties define this region of cII as that which interacts selectively with the DNA recognition sequence, TTGCN₆TTGC.

Mutations in the proposed first helix identified the region important for nonspecific DNA binding

We have characterized several different amino acid substitutions in the proposed first α -helical DNA binding region of the cII protein (Figs. 3 and 4B). Analogous to the mutants discussed above, all of these variant proteins form tetramers, yet functionally, some of them are 'up' mutations and some are 'down' mutations.

In an attempt to obtain 'change of specificity' mutations in the cII protein that would recognize and function at an altered cII-dependent promoter signal, the cII recognition sequence at the P_{RE} promoter was changed to TTGTN₆TTGT. This defective promoter failed to

A

					30					35					40					45
λ c11	THR	GLU	LYS	THR	ALA	GLU	ALA	VAL	GLY	VAL	ASP	LYS	SER	GLN	ILE	SER	ARG	TRP	LYS	ARG
λ c1	GLN	GLU	SER	VAL	ALA	ASP	LYS	MET	GLY	MET	GLY	GLN	SER	GLY	VAL	GLY	ALA	LEU	PHE	ASN
λ CRO	GLN	THR	LYS	THR	ALA	LYS	ASP	LEU	GLY	VAL	THR	GLN	SER	ALA	ILE	ASN	LYS	ALA	ILE	HIS

B

Figure 4. (A) Alignment of cII amino acid sequences in the region (from amino acid position 26 to 45) corresponding to α -helices 2 and 3 of λ repressor and λ cro protein. Regions of amino acid identity are boxed. (B) Amino acid sequence and single amino acid substitutions in the proposed helix-turn-helix region of the λ cII protein [amino acid positions 26–45]. The amino acid alterations shown below the sequence are defective in cII function. Those shown above the sequence indicate mutations that maintain full (solid vertical lines) or partial (dotted vertical lines) cII function.

Table 2. Native molecular forms and stabilities of cII and cII mutants

Name	Codon of amino acid substitutions	Native molecular form ^a	Half-life (min)
cII		tetramer (1, 2, 3)	15
Can1 cII	2 (Val → Ala)	tetramer (1, 2, 3)	49
3059ctrl	2, 4 (Val → Asp, Ala → Thr)	tetramer (3)	
3073	2 (Val → Leu)	tetramer (3)	
3107	7 (Arg → Cys)	tetramer (3)	10
3085	9 (Glu → Gly)	monomer (3)	0.8
2002	24 (Leu → Pro)	monomer (3)	2.5
3606	28 (Lys → Glu)	tetramer (3)	7
DYA-1	31 (Glu → Lys)	tetramer (3)	10
3641	32 (Ala → Ser)	tetramer (3)	3
3612	34 (Gly → Ser)	tetramer (3)	3
3563	37 (Lys → Arg)	tetramer (3)	15
SDM-1	37 (Lys → Glu)	tetramer (3)	15
3062	38 (Ser → Leu)	tetramer (1)	15
3283	40 (Ile → Asn)	tetramer (3)	14
3378	41 (Ser → Gly)	tetramer (3)	15
3151	42 (Arg → Gly)	tetramer (3)	15
3133	46 (Asp → Gly)	tetramer and monomer (3)	
3600	55 (Leu → Phe)	monomer (3)	0.7
3500	67 (Met → Thr)	monomer (3)	4
3331	70 (Leu → Phe)	monomer (3)	0.6
3192	70 (Leu → Ser)	monomer and a small amount of tetramer (3)	1
3639	92 (Gln → Arg)	tetramer (3)	4.5
3638	95 (Met → Val)	tetramer (3)	5

^a Determination methods are indicated in parentheses: (1) Velocity sedimentation with purified protein; (2) sedimentation equilibrium with purified protein; (3) velocity sedimentation with [³⁵S]methionine-labeled crude extract.

function in the presence of wild-type cII, and cII mutants were selected that allowed this signal to function efficiently (Wulff and Mahoney 1987). Two different mutations were obtained repeatedly and were characterized to be Glu-27 to Lys (designated as dya-8) and Glu-31 to Lys (designated as dya-1) within the proposed first α -helical region. Surprisingly, both mutant cII proteins not only functioned at the defective promoter but also retained their ability to activate the wild-type promoter. These results suggested that the two mutants had, in fact, not altered the DNA sequence-recognition specificity of cII but, rather, had overcome the defect in promoter recognition by some other mechanism. To examine the biochemical basis for this, we overproduced and purified the dya-1 protein. Examination of the chromatographic ability of this protein on DNA-agarose columns demonstrated that the protein exhibited a very strong nonspecific interaction with DNA. Similarly, very high salt concentrations were required to elute this protein during phosphocellulose chromatography (0.68 M of salt for dya-1 as compared with 0.46 M salt for wild-type cII). Moreover, in both in vitro transcription reactions and

DNA footprinting experiments, this protein exhibited a dramatically enhanced (approximately eightfold) ability to bind and function at the P_{RE} promoter (Fig. 5). These results indicate that the mutant protein has dramatically increased nonspecific affinity for DNA, which allows it to recognize and function with enhanced activity at the wild-type signal and also to recognize and function at altered signals that would normally be defective.

Genetic experiments indicate that the Lys-28 to Glu and the Ala-32 to Ser or Val alteration in this same region results in a cII 'down' effect. We have expressed and purified the more severely defective Lys-28 to Glu protein. DNase protection experiments demonstrated that this protein is indeed defective in DNA interaction (Fig. 5A). Surprisingly, however, and in dramatic contrast to the mutations in the second α -helix described above, in the presence of RNA polymerase, this mutant exhibits its ability to bind selectively to the appropriate DNA recognition site (albeit at greatly reduced affinity; Fig. 5A). At concentrations of protein 10 times higher than wild-type cII, this mutant also activates transcription in vitro from the appropriate promoter signals (Fig. 5B). These results once again suggest that this region of cII plays a major role in determining the general affinity for DNA rather than its sequence-specific DNA interaction. The nature of the amino acid substitution in this cII variant is most consistent with this contention. The lysine residue at these positions would be expected to favor ionic interaction between cII and DNA, whereas the acidic glutamate substitution would oppose this interaction. Clearly, the results from these mutational analyses support the contention that cII utilizes the helix-turn-helix motif for DNA interaction. In addition, they strongly implicate the first α -helical region as having a primary function in stabilizing the nonspecific interactions of the protein with DNA via ionic interactions with the phosphate residues along the backbone of DNA. Mutations in other DNA-binding proteins that exhibit increased DNA binding affinities have been reported (Betz and Sadler 1976; Pfahl 1979, 1981; Kelley and Yanofsky 1985; Nelson and Sauer 1985).

The oligomerization domains and their importance for protein stability

The native form of the wild-type cII protein is a 44-kD tetramer comprised of four identical subunits (Ho et al. 1982). Determination of the molecular weight of the native form of cII originally required sufficient pure protein to allow both velocity centrifugation and equilibrium sedimentation analysis. We have now developed a relatively simple procedure for determining the molecular form of cII and the various cII mutant proteins without requiring their extensive purification from cell lysates. The procedure can be summarized as follows: [³⁵S]methionine pulse-labeling of the induced cells that carry the appropriate expression vector containing the cII variant gene, cell lysis, salt extraction of protein, removal of high-molecular-weight nucleic acids by DEAE

Figure 5. (A) DNase protection experiment comparing the binding of wild-type cII and mutant cIIs. DNase I protection experiments were carried out essentially as described previously (Galas and Schmitz 1978; Ho et al. 1983). A 540-bp *Ava*I–*Bgl*II restriction fragment [nucleotide positions 38, 215–38, 754 of phage λ , as determined by Sanger et al. 1982] carrying the P_{RE} promoter was 5′-end-labeled with ³²P at the *Ava*I site. All reactions contained 0.1 pmole of this DNA fragment dissolved in 50 μ l of 50 mM Tris–HCl (pH 8.0) 80 mM KCl, 8 mM MgCl₂, 3 mM CaCl₂, 2 mM DTT, and 5% glycerol. Purified cII and cII mutant proteins were added as indicated. The relative units of protein are given at the top of each lane. One unit corresponds to a concentration of 0.35 μ M tetramer. Where indicated, 0.6 unit of RNA polymerase (sp. act. 320 μ /mg; Molecular Biology Division, Pharmacia) was added to the reaction. Samples were incubated at 23°C for 20 min. DNase I (4 ng) was added, and incubation at 23°C was continued for 2 min. The reaction was stopped by adding 50 μ l of a solution containing 0.6 M NaOAc, 100 mM EDTA, and 120 μ g/ml carrier tRNA, followed immediately by an equal volume of phenol. The mixture was extracted twice with phenol, then twice with either, and finally precipitated with ethanol. The precipitate was redissolved in 8 μ l of an 80% formamide gel-loading buffer, heated at 95°C for 1 min, and 4 μ l was applied to a standard 8% DNA sequencing gel. (B) Transcription activation experiment comparing the activity of wild-type cII and mutant cIIs. Transcription reactions were carried out as described previously using a plasmid that carries P_{RE} promoter signal as DNA template (Ho et al. 1982). An autoradiogram is shown of a PAGE analysis of ³²P-labeled RNA transcripts synthesized in vitro in the presence of purified cII proteins. The relative units of protein are given at the top of each lane. One unit corresponds to a concentration of 45 nM tetramer. (1) No cII protein; (2) wild-type cII; (3) cII Val-2 to Ala; (4) cII Lys-28 to Glu; (5) cII Glu-31 to Lys; (6) cII Lys-37 to Glu; (7) cII Lys-37 to Arg; (8) cII Ser-38 to Leu; (9) cII Ser-41 to Gly; (10) cII Arg-42 to Gly.

chromatography, and, finally, velocity sedimentation analysis of the extracted proteins using a 17–35% linear glycerol gradient. The position of the cII derivative within the gradient is determined by immunoprecipitation across the gradient using a high-titer rabbit antiserum made against purified wild-type cII protein. This polyclonal antiserum recognizes all of the single amino acid variants of the cII protein studied to date.

The procedure was developed and standardized using both the wild-type cII protein as well as a second functional cII derivative containing a single amino acid substitution at the second codon position, the *can1* cII mutant (Jones and Herskowitz 1978; Wulff et al. 1980). The molecular weights of both of these proteins deter-

mined from cell extracts were identical to that determined previously using purified protein (Ho et al. 1982; Y.S. Ho and M. Rosenberg, unpubl.). Similar analyses were carried out on a variety of the cII single amino acid variants, and representative examples of the gradient analysis are shown in Figure 6 and the results are summarized in Table 2.

Most of the mutations fell into two distinct classes based upon their sedimentation properties. One group sedimented identically to wild-type cII and thus retained its tetrameric conformation. In contrast, the second class sedimented near the top of the gradient, at the position expected for the monomeric 11,000-dalton form of the protein. Apparently, this second group of mutants

represents amino acid alterations that affect the ability of the protein to oligomerize to the tetrameric form. Most interestingly, these mutants clustered within two distinct regions of the protein, extending between amino acid residues 9 and 25 and between amino acids 46 and 71. All of these mutants (i.e., 14 different substitutions between positions 9 and 25, and 19 different substitu-

tions between positions 46 and 71) failed to function (i.e., exhibit a cII⁻ phenotype). This suggests that the defectiveness of these particular proteins may result directly from their inability to form tetramers. These are the only two regions of the protein that exhibit this correlation between function and ability to oligomerize. We conclude that these mutants define two regions of the cII protein that serve primarily a structural role in achieving formation of the active tetrameric form of the protein, either directly through the alteration of amino acids required for contacts between subunits or indirectly through the alteration of amino acids required for the formation of the proper conformation of the subunit itself. Interestingly, these two domains that comprise 40% of the molecule contain more than 60% of the hydrophobic residues of cII, implying that hydrophobic interfaces might be involved directly in the subunit-subunit interaction in the tetrameric conformation.

During the analysis of the various cII mutants, it was noted that an unexpectedly large class of the variants was far less stable in the *E. coli* host than wild-type cII is. Pulse-chase experiments were used to examine and compare the half-lives of the various cII proteins more precisely. The results (representative examples of the pulse-chase analysis are shown in Fig. 7 and summarized in Table 2) indicate that one class of mutants in particular exhibits a half-life of less than 3 min (some as low as 36 sec), as compared with the 15-min half-life for wild-type cII. Most remarkably, these highly unstable variants are those that are unable to form tetramers. In contrast, those cII mutants that had stabilities comparable to wild-type all retained the tetrameric conformation. Apparently, the inability to form a tetramer is an important parameter of cII stability, implying that either the monomeric form of cII is highly accessible to proteolytic degradation or the mutations prevent the monomer from folding, thereby indirectly preventing the formation of tetramers.

Figure 6. Fluorograms of cII and cII mutant proteins resolved on SDS-polyacrylamide gels after velocity sedimentation analysis. Crude cellular extracts of cII or cII mutant protein, pulse-labeled with [³⁵S]methionine in vivo, were subjected to the glycerol gradient ultracentrifugation analysis as described in Methods. A total of 18–19 fractions were collected from each sample, precipitated by anti-cII antibody, resolved on 15% SDS-PAGE, and visualized by fluorography (see Methods for details). The native molecular forms of cII mutant proteins were determined by comparing their velocity sedimentation positions with wild-type cII and with the molecular weight protein standards, analyzed separately on glycerol gradients as described in detail by Ho et al. (1982). [A] Wild-type cII; [B] cII 2002 (Leu-24 to Pro); M the crude cellular extract of cII 2002 used as the marker; [C] cII 3639 (Gln-92 to Arg). The five standard proteins have sedimentation coefficients and molecular weight, respectively, as follows: lysozyme 1.91S and 14,400; soybean trypsin inhibitor 2.3S and 24,000; carbonic anhydrase 3.0S and 34,000; ovalbumin 3.66S and 45,000; bovin serum albumin 4.3S and 67,000 (Edsal 1953).

Figure 7. [³⁵S]Methionine pulse-chase analysis comparing the protein stability of wild-type cII and mutant cIIs. (A–C) Autoradiograms of pulse–chase experiments of wild-type cII, cII mutants Glu-9 to Gly and Arg-42 to Gly (see Methods for details). The first lane of each autoradiogram is the control, which has been pulse-labeled for 1 min at 32°C instead of at 42°C and chased for 5 min. The chasing times are given at the top of each lane. The migration positions of cII proteins are indicated by arrows. (D) Half-log plot of representative examples of the half-life determination of cII and various cII mutants. All of the mutants in the second α -helical DNA-binding region, including Lys-37 to Glu, Lys-37 to Arg, Ser-38 to Leu, Ile-40 to Asn, Ser-41 to Gly, and Arg-42 to Gly, have the same half-lives as wild-type cII protein. The mutants in the region of amino acid residues 9–25 and 46–71, including Glu-9 to Gly, Leu-24 to Pro, Leu-55 to Phe, Met-67 to Thr, Leu-70 to Ser, and Leu-70 to Phe, are very unstable. Their half-lives fall in the same category as Glu-9 to Gly and Leu-55 to Phe, which are shown here as two representative mutants of these two regions.

Recently, we have found an *E. coli* host strain that stabilizes this particular class of highly unstable cII mutant. In this host strain [HtpR[−] (high temperature production regulator)] all the highly unstable cII mutants exhibit stabilities comparable to or greater than wild-type cII. For example, the highly unstable cII mutant, cII 3085 (Glu-9 to Gly), which exhibits a half-life of less than min in a normal host (Fig. 8A), exhibits a half-life of at least 20 min in the HtpR[−] host (Fig. 8B). Although this protein now accumulates in the HtpR[−] background, it still does not form a tetramer when assayed by velocity sedimentation. Apparently, the host mutant effect

is simply to stabilize the monomeric form of the protein against proteolytic degradation. These results strengthen our previous suggestion that these two regions comprise structural domains crucial for stable tetramer formation.

It is of interest to note that although the HtpR[−] host mutant stabilized these cII variants, it has no effect on the stability of wild-type cII (Fig. 8C,D). Because the HtpR gene of *E. coli* encodes a sigma factor, σ^{32} , that promotes transcription from heat shock promoters, the HtpR[−] cell line is defective in heat shock response (Grossman et al. 1984, 1985). Our results indicate that a

Figure 8. $[^{35}\text{S}]$ Methionine pulse-chase analysis, comparing the protein stability of a highly unstable cII mutant, Glu-9 to Gly with wild-type cII in the HtpR⁻ and HtpR⁺ *E. coli* host strains (AR67 and AR65, respectively). The experimental details are the same as those described for Fig. 5A. The autoadiograms of pulse-chase experiments are shown as follows: (A) cII Glu-9 to Gly in HtpR⁺ (AR65); (B) cII Glu-9 to Gly in HtpR⁻ (AR67); (C) wild-type cII in HtpR⁺ (AR65); (D) wild-type cII in HtpR⁻ (AR67).

proteolytic activity under the control of the heat shock system might be responsible for the rapid degradation of these highly unstable cII mutants. Recently, several foreign gene products, including both prokaryotic and eukaryotic, have been stabilized and overproduced in this mutant strain (European patent application 86870137.6), which suggests that this proteolytic activity can discriminate mutated, foreign, or otherwise altered forms of proteins from the normal *E. coli* proteins. The conclusion that different degradation mechanisms exist for the turnover of mutated and normal cII proteins is further supported by the observation that those phage and host functions (cIII and Hfl, respectively; Herskowitz and Hagen 1980; Wulff and Rosenberg 1983) that affect the stability of wild-type cII have no effect on any of the mutant proteins (Y.S. Ho and M. Rosenberg, data not shown).

Finally, although we have demonstrated that cII quaternary structure is necessary for both cII stability and function, it is not sufficient. As we have described above, the cII mutants mapping in the specific DNA-binding domain retain their ability to form tetramers but have lost both their transcription activation and DNA-binding functions.

The amino-terminal domain

Fifteen different single amino acid substitutions have now been characterized in the seven amino acid codons positioned between residues 2 and 8 of the cII protein (Fig. 3). Most unexpectedly, the majority of these mutants exhibit a cII⁺ phenotype and cannot be distinguished either biochemically or genetically from cII wild-type. One of the cII⁻ derivatives found in this region has been shown previously to affect the translation efficiency of the gene (Place et al. 1984), rather than its biochemical function. The only cII⁻ mutation that occurs in these seven amino-terminal residues and causes a biochemical defect in the protein is the alteration of Lys to Glu at position 6. Other mutations that alter this same Lys-6 residue have no effect on cII function and, thus, indicate that the lysine residue per se is not critical for function. Instead, it appears that only the particular substitution of the basic lysine with an acidic residue is unacceptable for cII function. These results strongly suggest that the amino-terminal region of the cII protein has an unusual flexibility in its specific amino acid sequence. This amino-terminal flexibility is different from that found in the amino-terminal region

of the λ repressor (cI), which is known to be very important for DNA binding (Eliason et al. 1985). It is of interest to note that cII binds selectively to DNA with a natural affinity that is approximately two orders of magnitude lower than that of the λ repressor (Johnson et al. 1979). We assume that part of the difference in DNA affinity exhibited by these two proteins can be accounted for by the properties of their respective amino-terminal regions.

The carboxy-terminal domain

The last domain of the cII protein encompasses the carboxy-terminal 25-amino-acid residues (Fig. 3). Of more than 500 cII⁻ mutants isolated by Wulff and his co-workers (1984), no amino acid substitution mutant mapped to the right of the cII 3139 mutation (Leu-78 to Pro) at codon 78, although a restricted search for partially active mutants yielded two mutations near the carboxy-terminal end (Gln-92 to Arg and Met-95 to Val) that retain considerable cII activity. Apparently the carboxy-terminal end of the protein is underrepresented in the analysis at ordinary cII⁻ mutations because most amino acid substitutions in this region do not affect protein function severely. Two mutant proteins with different amino acid substitutions in this domain (Table 2) were found to be primarily in the tetrameric form. Pulse-chase experiments demonstrated that these mutants are significantly less stable in *E. coli* than wild-type cII is, although more stable than the highly unstable mutants described earlier (Table 2). Because the highly unstable mutants were unable to form tetramers, perhaps these mildly unstable mutants were only partially impaired. Consistent with this idea, we found that this carboxy-terminal mutant class was also stabilized in the HtpR⁻ host background analogous to the highly unstable mutants. For example, the half-life of the Gln-92 to Arg mutant was extended from 4.5 min in the normal host to 45 min in the HtpR⁻ host. Possibly these mutants are recognized by the same proteolytic system that degrades the highly unstable class of cII mutants; but they do not appear to be as susceptible to this system, presumably because they still form tetramers. We assume that the carboxyl-terminal domain is playing primarily a structural role in helping to stabilize the formation of the active cII tetramer but is not critical to tetramer formation nor essential for DNA interaction.

Methods

Bacterial strains

The bacterial lysogens used in this work are all made in *E. coli* K12 strains designated as follows: N99(λ cI857), AR67: CAG456(λ cI857 kil-H1)galE::Tn10, (patent 14252); AR65: SC122(λ cI857 kil-H1)galE::Tn10 (patent 14252), and UC5822: N99(λ int 6 red 3 cI1857 cII3067 Pam 3).

Plasmid constructions

The structural genes of the mutant cII proteins were cloned into the *E. coli* expression vector system pKC30, as described by Shimatake and Rosenberg (1981), for the construction of the

expression system of wild-type cII. The following change was made to eliminate the λ O-protein coding sequence from the DNA fragment. A 603-bp *Hae*III–*Bgl*II fragment containing the structural gene of each single amino acid cII mutant was inserted into the pKC30 vector, cut with *Hpa*I and *Bam*HI, which put the gene directly under the transcriptional control of the inducible phage λ P_L promoter. Because O protein is always highly expressed in the pKC30 system, this change greatly facilitates the protein purification procedure.

All plasmid constructions were analyzed by restriction analysis, and all methods used in construction of these vectors were standard procedures described by Maniatis et al. (1982).

Protein purification

The purification of the mutant cII proteins was carried out as described previously for the wild-type cII purification (Ho et al. 1982), except for the following two cases. cII 3062 did not bind to the anion exchanger column used for the wild-type cII purification. Instead, it was purified by a cation exchanger chromatography (Tris acryl CM ion exchanger from LKB). Dya-1 binds strongly to the chromosomal DNA, so it was first treated with 1% polymin P to precipitate the nucleic acid and then purified by Whatman P11 phosphocellulose chromatography.

Pulse-chase experiment

Cells were grown overnight at 32°C in modified minimal media M56 (Gottesman and Yarmolinsky 1968) with ampicillin (50 μ g/ml) from a fresh single colony. The next morning, the cell culture was reinoculated into the same medium with 1 : 10 dilution ($A_{650} = 0.15$) and grown at 32°C until $A_{650} = 0.6$. An aliquot of cell culture, usually 2.0 ml, was harvested by centrifugation at 3000 rpm for 15 min at room temperature. The cell pellet was resuspended in half volume (i.e., 1 ml) of minimal media M56, supplemented with 0.2% glucose, 0.2 mM amino acid mixture lacking methionine, 1 μ g of thiamine, and 50 μ g of ampicillin per milliliter and incubated at room temperature for 30 min. Equal volume (i.e., 2 ml) of the same media, prewarmed to 65°C, was added to raise the temperature of the cell suspension quickly to 42°C and incubated in a 42°C water bath. Twenty minutes later, the cells were pulse-labeled with 200 μ Ci of [³⁵S]methionine for 1 min ([L-³⁵S]methionine, 600 Ci/mmol; Amersham) and chased by adding into an equal volume (i.e., 2 ml) of 2 mM nonradioactive methionine together with chloramphenicol at a final concentration of 250 μ g/ml to stop de novo protein synthesis. A 0.4-ml aliquot was taken out at different time points as indicated in the figure legend (Figs. 7 and 8) and added into an Eppendorf tube containing 60 μ l of 20% SDS and 35% of β -mercaptoethanol. The samples were heated at 95°C for 1 min, followed by quick freezing in a dry ice-ethanol bath, and precipitated with trichloroacetic acid (final concentration 10%). The precipitates were rinsed twice with cold ethanol, resuspended in sample buffer [60 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β -mercaptoethanol, 2% SDS, and bromophenol blue], heated at 95°C for 5 min, and subjected to electrophoresis on a 15% polyacrylamide gel. Finally, the gels were dried and fluorographed (Bonner and Laskey 1974). The intensities of the cII protein bands were quantitated by using a Beckman DU-8 spectrophotometer with a gel-scanning attachment. All of the pulse-chase experiments were done in the UC5822.

Native molecular weight determination of proteins

A simple procedure was developed for determining the native molecular weight of a protein, without requiring high-level ex-

pression and extensive purification of material from cell lysates. The procedure is summarized as follows: cII protein was pulse-labeled in vivo with [³⁵S]methionine as described for 5 min. The cells were collected by microcentrifugation, frozen and thawed three times to break the cell wall, and then treated with lysozyme with a final concentration of 0.25 mg/ml at 0°C for 20 min. After this step, the cell lysate was sheared at high speed for 30 sec with a Tekmar model TR-10 homogenizer. The cII protein was extracted by 0.3 M KCl [in 50 mM Tris-HCl (pH 8.0) with 0.1 mM EDTA and dithiothreitol (DTT)] with continuous stirring at 0°C for 1 hr, followed by high-speed centrifugation at 32,000 rpm for 30 min. The supernatant was collected and passed through a 1-ml bed volume DEAE-Sephacel column to clean away the DNA from the sample. The column was originally equilibrated and eluted after the loading of the sample with the same buffer as described for salt extraction. The eluant was monitored by a Geiger counter. The fractions with the highest radioactivities were collected and pooled. Then, 0.2 ml of the [³⁵S]methionine-labeled cII sample was layered on the top of a 17–35% linear glycerol gradient (total volume of the gradient was 4 ml) and centrifuged in a Beckman swinging-bucket rotor SW 60 at 54,000 rpm for 42 hr (Ho et al. 1982). Finally, 18–19 fractions were collected from each sample by bottom punctuation. Each fraction was immunoprecipitated with anti-cII antibody (as described below) and resolved on 15% SDS-PAGE. The cII bands on the gel were visualized by fluorography.

Immunoprecipitation analysis

An aliquot of 100 µl was removed from each fraction collected from the velocity sedimentation experiment and mixed with 10 µl of preimmune rabbit serum and 50 µl of formalin-fixed Staph A cells (from BRL). After incubation at 0°C, the supernatant was collected by microcentrifugation and mixed gently with 2 µl of rabbit anti-cII antiserum purified by DEAE-cellulose [(DE-52 column, Whatman) (Ho et al. 1982)] After incubation at 4°C overnight, 50 µl of Staph A cells were added into each sample and incubated at 0°C for 1 hr. The immune complexes were collected by a quick spin (30 sec) on microcentrifuge and washed repeatedly (usually six times) with phosphate saline at pH 7.2 containing 10% (wt/vol) β-mercaptoethanol and 3% (wt/vol) SDS with vortex and quick spin following each wash. After the final wash, the pellets of the immune complexes were air-dried by inverting the tubes on a lab bench for 10–20 min. They were resuspended in 40 µl of sample buffer, resolved by 15% SDS-PAGE, and prepared for autoradiography using fluorographic amplifying reagent enhancer, as described by the manufacturer (New England Nuclear).

Transcription assays

Transcription reactions were carried out as described previously, using a plasmid that carries P_{RE} promoter signal as DNA template (Ho et al. 1982). The amounts of purified cII proteins added to each reaction are indicated in the figure legend.

DNase I protection experiments

The DNase I protection experiments were carried out as described previously (Galas and Schmitz 1978; Ho and Rosenberg 1983). In all cases, the reactions contained 0.02 pmole of the 5'-end-labeled DNA fragment containing the P_{RE} promoter signal (Ho et al. 1983). The amounts of cII proteins and RNA

polymerase added to each reaction are indicated in the legend to Figure 5A.

Site-directed mutagenesis

The site-directed mutagenesis for changing the codon AAG (Lys-37) to GAG (Glu) was carried out following the methods described by Zoller and Smith (1983). A short oligonucleotide 3'-CAACTACTCAGCGTC-5' was synthesized by Dr. G. Sathe for directing the mutagenesis. The desired mutation was verified by M13 sequencing (data not shown).

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Identification of the DNA binding domain of the phage lambda cII transcriptional activator and the direct correlation of cII protein stability with its oligomeric forms.

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