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Biochemical characterization of L1 repressor mutants with altered operator DNA binding activity

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A mycobacteriophage-specific repressor with the enhanced operator DNA binding activity at 32°C and no activity at 42°C has not been generated yet though it has potential in developing a temperature-controlled expression vector for mycobacterial system. To create such an invaluable repressor, here we have characterized four substitution mutants of mycobacteriophage L1 repressor by various probes. The W69C repressor mutant displayed no operator DNA binding activity, whereas, P131L repressor mutant exhibited very little DNA binding at 32°C. In contrast, both E36K and E39Q repressor mutants showed significantly higher DNA binding activity at 32°C, particularly, under in vivo conditions. Various mutations also had different effects on the structure, stability and the dimerization ability of L1 repressor. While the W69C mutant possessed a distorted tertiary structure, the P131L mutant dimerized poorly in solution at 32°C. Interestingly, both these mutants lost their two-domain structure and aggregated rapidly at 42°C. Of the native and mutant L1 repressor proteins, W69C and E36K mutants appeared to be the least stable at 32°C. Studies together suggest that the mutants, particularly P131L and E39Q mutants, could be used for creating a high affinity temperature-sensitive repressor in the future.

Introduction

Mycobacteriophage L1, a temperate bacteriophage, possesses a 50 kb double-stranded DNA genome that can integrate into a specific site in the chromosome of *Mycobacterium smegmatis*.[1] Genetic studies indicated that L1 carries 28 genes for regulating lytic development and a repressor (*cI*) gene for maintaining lysogeny.[2] The *cI* gene was cloned, sequenced and found to encode a protein of 183 amino acid residues.[3] Interestingly, L1 repressor (CI) shares ~40–100% identity with those of other temperate mycobacteriophages (i.e., L5, Bxb1, MS6 and Bxz2) at the amino acid sequence level.[3,4] Except for the N-terminal end region, none of the mycobacteriophage-specific repressors exhibited significant homology with those of the lambdoid phages,[5,6] indicating that repressors encoded by these phages belong to a new family of negative transcriptional regulators.[3] Despite the dissimilarity at the primary structural level, the L1 CI monomer[7] possesses a two-domain structure (an N-terminal domain, a C-terminal domain and a hinge region linking the two domains) like that of the λ phage repressor monomer.[8] The N-terminal and the C-terminal domains of L1 CI were also suggested to possess similar functions as their counterparts in the λ repressor.[8] The N-terminal domain (NTD), which is composed of amino acid residues from ~1–54, carries a putative helix-turn-helix (HTH) DNA-binding motif, whereas, the C-terminal domain (CTD) that harbors amino acid residues from ~63–183 was suggested to be involved in the dimerization of CI in solution. Both CI and CTD contain a significant amount of α-helices at 30°C.[7] Interestingly, the concentration of CI in a L1 lysogen is similar to that of a λ lysogen.[6,9]

Despite similarities at the domain structure and functional level, CI binds with dissimilar affinity to two asymmetric operator DNAs (designated O_{64} and O_L).[7] While O_{64} carries the putative operator sequence 5'GGTGGATGTCAAG, O_L harbors the sequence 5'GGTGGCTGTCAAG. Repressor proteins of mycobacteriophages L5 and Bxb1 also showed binding to multiple asymmetric operator DNAs.[4,10] Interestingly, sequences of L1 operator DNAs showed 100% identity with those of L5 and moderate identity with those of Bxb1.[3,4,11] Operator DNA binding activity of CI at 42°C was less than that at 32°C.[12] Other physical factors that strongly influenced the structure and function of CI are the ions and the ionic strength of solution.[13,14] Of the various monovalent and polyvalent ions, Na^+ was suggested to promote the optimal binding of the L1 repressor to the operator DNA.[14] Recently, several bases, two adjacent major grooves and one face of the 13 bp operator DNA helix were reported to be crucial for CI binding.[9] Unexpectedly, CI that induced a little bending in the operator DNA exhibited binding as a monomer.

To study the expression and function of mycobacterial genes in the homologous environment, several expression vectors were developed in the past 15 y.[15-21] All of these vectors require different chemical inducers (like acetamide, tetracycline, pristinamycin, etc.) for initiating the desired gene expression. Most of

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these vectors, however, have a number of drawbacks including the leakiness in the absence of inducer. Previously, numerous expression vectors were generated by exploiting the temperature-sensitive repressor genes and the early promoters of the lambdoid phages.[22-24] The uniqueness of such vectors is that they do not require any chemical inducer and employ only temperature shifts to turn on/off the gene expression from the early promoters. Although the repressor-controlled early promoters and the temperature-sensitive repressor genes were reported from both mycobacteriophages L1[2,3,11] and L5,[10,25] they haven't been assembled for generating a temperature-inducible mycobacteria-specific expression vector. Apparently, the reasons why the above gene regulatory elements of L1 / L5 were not fused in creating an expression vector are the relatively weaker operator DNA binding affinity of L1 / L5 repressor and the limited repression of transcription from the early promoter P_{left} in vivo.[7,10,11] In addition, the operator DNA binding affinity of any temperature-sensitive L1 or L5 repressor is yet to be investigated systematically.

Several mutant repressors of phages λ, 434 and P22, which harbored acidic or non-polar to basic amino acid substitution in the HTH (helix-turn-helix) motifs and neighboring regions, were shown to possess higher operator DNA binding activity than the corresponding wild-type counterpart.[26-28] Thus far, no high affinity mutant of L1 or L5 repressor was generated or screened that possesses superior operator DNA binding activity than the wild-type repressor. In the present work, we have studied four mutants of L1 repressor and demonstrated that E36K and E39Q mutant repressors augmented, whereas, W69C or P131L mutant repressor completely or partially abolished the operator DNA binding activity of L1 repressor at 32°C. Structures of the latter two mutants were severely affected at 42°C. Interestingly, W69C and E36K mutants appeared to be less stable than the other mutant and the wild-type repressors.

Results and Discussion

Operator DNA binding affinity of different L1 repressor mutants in vivo. To accelerate the construction of a temperature-inducible expression vector for mycobacterial systems, four L1 repressor mutants (namely, CIE39Q, CIE36K, CIW69C and CIP131L) were expressed and characterized in detail (**Table 1**). CIE36K and CIE39Q, which harbor Glu to Lys and Glu to Gln substitutions at the positions 36 and 39, may bind the operator DNA relatively tightly as these mutations decreased the total negative charge of the putative HTH motif of CI.[3] In contrast, CIP131L, carrying a Pro to Leu substitution at the 131st position of CI, is a temperature-sensitive mutant possessing operator DNA binding activity at 32°C but not at 42°C.[2,12] Previously, Trp residue at the 69th position was not recognized by chymotrypsin since the 70th residue of CI is a Pro residue.[3,7] To precisely determine the status of Trp69 in CI, we initially constructed CIW69C (by replacing Trp with Cys at the 69th position) and found that its Cys residue is exposed (data not shown), indicating the surface exposure of Trp69 in CI. Additional experiments (such as X-ray crystallography or NMR spectroscopy), however, need to be performed to verify the above hypothesis. Our preliminary investigation also suggested that CIW69C, like CIP131L, is a temperature-sensitive mutant of CI (data not shown and see below).

A temperature-sensitive λ repressor (such as CI857) has been successfully used in the generation of several expression vectors.[24] To verify whether CIW69C and CIP131L could function like that of λ CI857 and to determine whether CIE36K and CIE39Q possess superior operator DNA binding affinity in vivo, we constructed different recombinant *M. smegmatis mc²155* strains using *M. smegmatis mc²155* and pMV261[29] or a pMV261 derivative (**Table S1**). While the strain harboring pMV261 alone would allow the growth of an infected L1 phage, strains producing repressor proteins from the pMV261 derivatives would hinder the growth of this phage by blocking the transcription of its lytic genes. The latter strains may also repress the growth of L1 phage differently if their repressor proteins possess variable DNA binding affinity. To obtain a clear view on the in vivo activity of the mutant CI proteins, the plating efficiencies of LIcI$^-$ phage (a lytic L1 phage variant)[2] on all of the recombinant strains were determined both at 32° and 42°C (**Table 1**). The plating efficiency of LIcI$^-$ on S5039 or S5041 was significantly less than that on S5037 (all p values less than 0.05) at 32°C, indicating that CIE39Q and C1E36K possess higher operator DNA binding affinity than CI at this temperature (**Table 1**). However, at 42°C, these two mutant repressor proteins could not repress the growth of LI*cI*$^-$ like that of the CI. The phage growth

Table 1. DNA binding activity of different mutant L1 repressor proteins

M. smegmatis strains	Plasmid in the strains	Repressor expressed by the strains	Amino acid change in repressor (at position)	*Plating efficiency (%) of L1cI$^-$ at		$^†K_d$ at 32°C
				32°C	42°C	(μM)
S5036	pMV261	Nil	Nil	100	100	
S5037	p1269	CI	Nil	45.42 ± 2.99	50.26 ± 2.17	0.31 ± 0.02
S5038	p1271	CIP131L	Pro to Leu (131)	53.53 ± 2.3	95.31 ± 4.14	ND
S5039	p1274	CIE39Q	Glu to Gln (39)	37.31 ± 1.74	57.73 ± 1.85	0.25 ± 0.04
S5041	p1275	CIE36K	Glu to Lys (36)	38.36 ± 0.75	55.82 ± 1.7	0.38 ± 0.04
S5044	p1277	CIW69C	Trp to Cys (69)	100	100	ND

*Percent efficiency of plating of L1*cI*$^-$ on different *M. smegmatis* strains were determined relative to *M. smegmatis* S5036 at 32°C/42°C. The efficiency of plating equals to plaque forming unit on experimental bacteria/plaque forming unit on control bacteria. $^†K_d$ (repressor concentration resulting half-maximal binding) values were determined from the gel shift assay data (presented in **Figure 1**) according to Bandhu et al.[9] ND, not determined.

repressing activity of CIP131L was less than that of CI (p = 0.02) at 32°C. At 42°C, this CTD mutant repressor was found almost inactive. In contrast, the Trp mutant did not show any repressing activity at either temperature, indicating that it does not possess any operator DNA binding activity.

In vitro DNA binding activity of L1 CI mutants. To verify the binding affinity of the mutant repressor proteins in vitro, a gel shift assay was performed using the labeled O_{64} DNA and varying concentrations of the histidine-tagged mutant and the wild-type CI proteins. **Figure 1A** shows that His-CIW69C did not bind to the operator DNA, whereas, the rest of the mutants and the wild-type repressor bound to the operator DNA at 32°C. As expected from the previous study,[12] His-CIP131L lost the operator DNA binding activity at 42°C (data not shown). In contrast, His-CI, His-CIE36K and CIE39Q exhibited the operator DNA binding activity at 42°C as well. Using the scanned data from the gel shift assay picture, the K_d (repressor concentration yielding half-maximal binding) values for His-CI - O_{64} DNA, His-CIE36K - O_{64} DNA and His-CIE39Q - O_{64} DNA interactions at 32°C were determined to be 0.31 ± 0.02, 0.38 ± 0.04 and 0.25 ± 0.04 μM, respectively (**Table 1**). The operator DNA binding capacity of His-CIP131L did not reach to 50% under the conditions of the assay. Half of the input operator DNA was also not bound by either His-CI or His-CIE36K at 42°C. Notably, we did not raise the repressor concentration more than 1 μM in the gel shift assay, as all of the proteins started non-specific DNA binding at concentrations ≥ 1.2 μM (data not shown). To get a comprehensive idea about the operator DNA binding affinity of all mutant repressor proteins, we compared the operator DNA binding affinity of each mutant repressor at 500 nM with that of the equimolar concentration of His-CI. As evident from **Figure 1B**, His-CIP131L exhibited about a 3 to 3.75-fold less operator DNA binding activity than His-CI, His-CIE36K and His-CIE39Q at 32°C. At 42°C, the operator DNA binding affinity of His-CIE36K was found ~2.75-fold less than that of His-CI or His-CIE39Q. Together, the in vitro operator DNA binding pattern of either His-CIP131L or His-CIW69C corroborates the corresponding in vivo data in **Table 1**. His-CIE39Q also appeared to possess marginally higher operator DNA binding affinity than His-CI at 32°C. It is not clear why His-CIE36K failed to yield superior DNA binding affinity in the gel shift assay at 32°C. As in vivo experiment partly corresponds to the lysogenic condition and was performed using proteins with no polyhistidine tag, the data yielded from such experiment are more trustworthy. The Glu to Lys and Glu to Gln substitution at positions 36 and 39 have likely augmented the operator DNA binding affinity of CI.

To see the binding specificities of CIP131L, CIE36K and CIE39Q, a DMS protection assay[9] was performed using labeled O_{64} DNA and His-CI, His-CIP131L, His-CIE36K and His-CIE39Q. The data revealed that the guanine bases protected by His-CI are also protected by all three mutant repressor proteins indicating that the binding specificities of the above mutant proteins remained unaltered (**Fig. 1C**).

Domain structure of the mutant repressor proteins. The altered DNA binding activities of the mutant L1 repressor proteins may be due to the alteration of the two-domain structure of CI. To confirm it, limited proteolysis[7,30,31] of all repressor proteins including His-CI were performed with chymotrypsin at 32°C and 42°C. Chymotrypsin was selected as it has been previously shown to produce two domains from His-CI.[7] The data show that all mutant proteins produced NTD- and CTD-specific fragments like that of His-CI at 32°C (**Fig. 2**). His-CI, His-CIE36K and His-CIE39Q also generated the domains-specific protein fragments at 42°C. However, His-CIE39Q was digested partially at 42°C while His-CIP131L and His-CIW69C remained mostly undigested. Collectively, substitution mutation at 36th position did not affect the two-domain structure of the CI, whereas, those at 39th, 69th and 131st positions affected it severely or partially, particularly at 42°C.

Aggregation of the mutant repressor proteins. Inhibition of the proteolytic digestion of His-CIW69C and His-CIP131L (**Fig. 2**) at 42°C may be due to the complete aggregation of these proteins at this temperature. In contrast, His-CIE39Q may have been aggregated partially as it showed some sensitivity to chymotrypsin (**Fig. 2**). To better understand the aggregation, absorbance values (at 360 nm) of all of the mutant repressor proteins and His-CI were recorded after transferring these protein samples from 32°C to 42°C. The absorbance values of all mutant proteins were increased more or less abruptly within ~1–5 min of shifting, whereas, that of His-CI was raised after 10 min of transfer (**Fig. 3**), indicating that aggregation of all mutant repressor proteins occurred prior to the aggregation of His-CI at 42°C. Compared with His-CIE39Q and His-CIE36K, both His-CIW69C and His-CIP131L also aggregated early and swiftly. Surprisingly, none of the above repressor proteins exhibited any aggregation at 32°C under the conditions of study (data not shown). The results together suggest that His-CIW69C and His-CIP131L are the most thermolabile proteins among those studied here. Rapid and possibly complete aggregation of His-CIW69C and His-CIP131L could have fully blocked the chymotrypsin-specific cleavage sites of these proteins at 42°C. In contrast, cleavage sites of other mutant repressor proteins were not totally masked despite their partial aggregation at 42°C.

Dimerization of the mutant repressor proteins. Similar to the λ repressor, wild-type L1 repressor was shown to form homodimeric molecules in solution.[7,8] Several mutations at the C-terminal domain affected both the dimerization and DNA-binding activity of λ repressor.[32-34] To see whether the substitution of Pro131 and other amino acid residues affected the dimerization of L1 repressor, crosslinking of all mutant repressor proteins and His-CI were performed in the presence of glutaraldehyde at 32°C. **Figure 4A** shows that dimerization of His-CIP131L molecules was severely affected in comparison with those by the equimolar concentrations of other repressor proteins including His-CI. **Figure 4B** reveals that the dimerization efficiency of His-CIP131L is about 2.5-fold less than that of His-CI (p = 0.015). The dimerization efficiencies of other mutant repressor proteins were not significantly altered at 32°C.

Structures of the mutant repressor proteins. To see the effects of mutations on the secondary and tertiary structures of L1 repressor, far-UV and near-UV CD spectra of all of the mutant

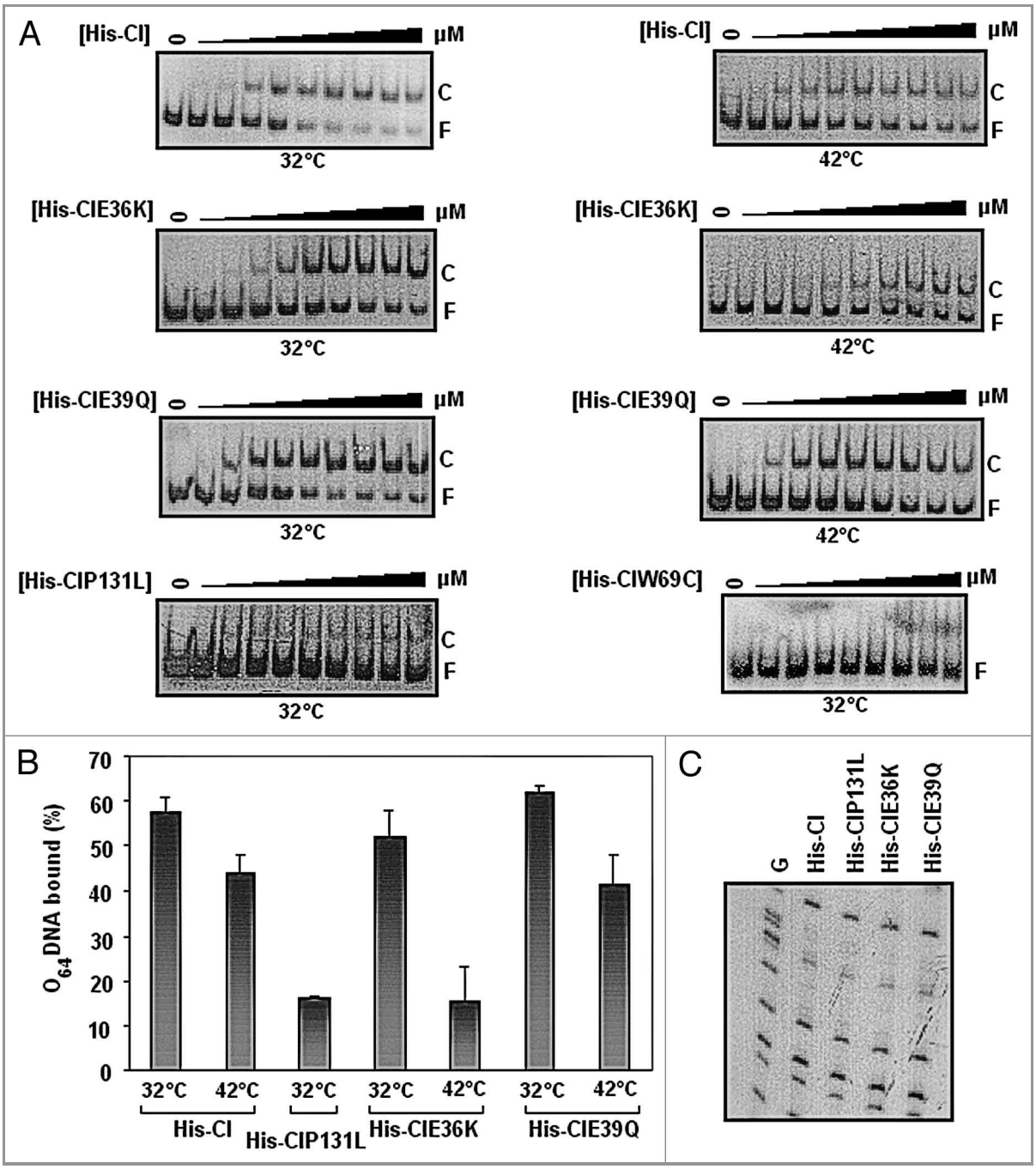

Figure 1. DNA binding activities of different repressors in vitro. (A) Autoradiograms of the gel shift assay using varying concentrations (~0.1–0.9 μM) of the indicated L1 repressor proteins and the ^{32}P labeled O_{64} DNA (0.1 nM) at 32°C and 42°C. C and F denote repressor-operator DNA complex and free operator DNA, respectively. (B) Bars denote the amount (%) of input O_{64} DNA bound by the indicated repressor proteins at 500 nM. Amount of operator DNA bound by each repressor at a specific temperature was determined from the corresponding gel shift assay data presented in panel A. The error bars indicate standard deviations of three independent experiments. (C) DMS protection assay using indicated repressor proteins and the ^{32}P labeled O_{64} operator DNA.

repressor proteins and His-CI were recorded at 32°C and compared. **Figure 5A** shows that far-UV spectra of all of the repressor proteins carry single peak with large negative ellipticity at 208 nm indicating the presence of α-helix in the proteins. The peak yielded by His-CIE36K possesses a marginally higher negative ellipticity value than those of equimolar concentrations of the other proteins. Analysis of the spectra with a software program CDNN,[35] however, indicates that amino acid substitution at the above positions does not significantly affect the α-helix content of CI at 32°C (data not shown). Near-UV CD spectra revealed that the tertiary structure of His-CIW69C is distorted in comparison with those of the other mutants and the wild-type CI at 32°C

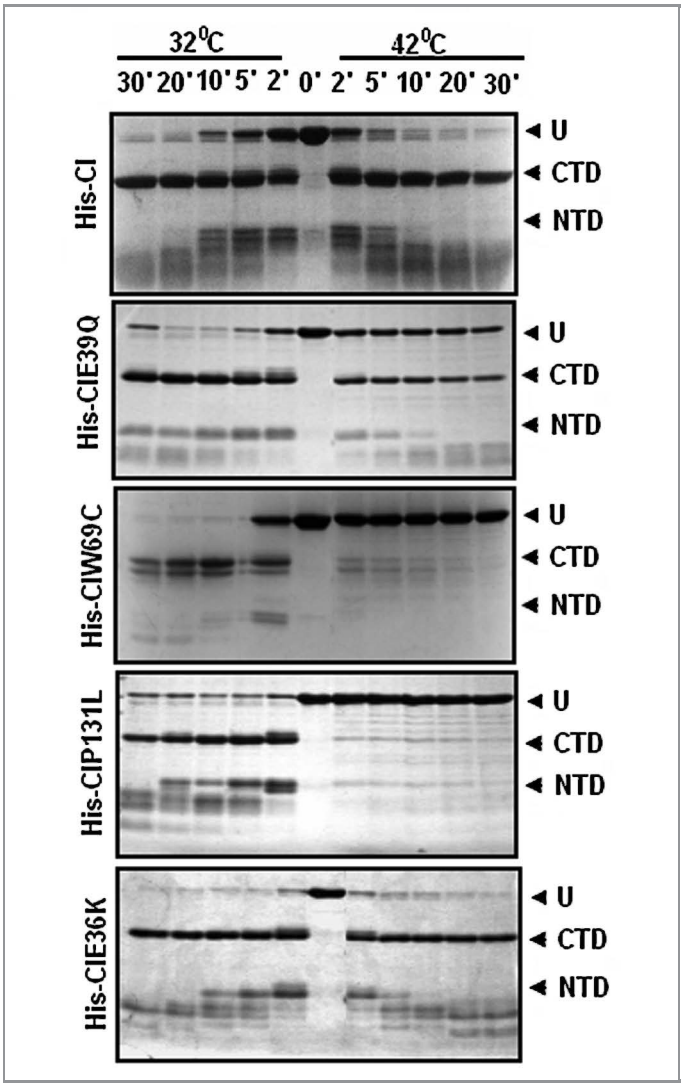

Figure 2. SDS-10% PAGE analysis of the proteolytic fragments. All indicated repressors were cleaved with chymotrypsin for 0'-30' min at 32°C and 42°C. U, CTD and NTD indicate undigested, C-terminal domain and N-terminal domain, respectively.

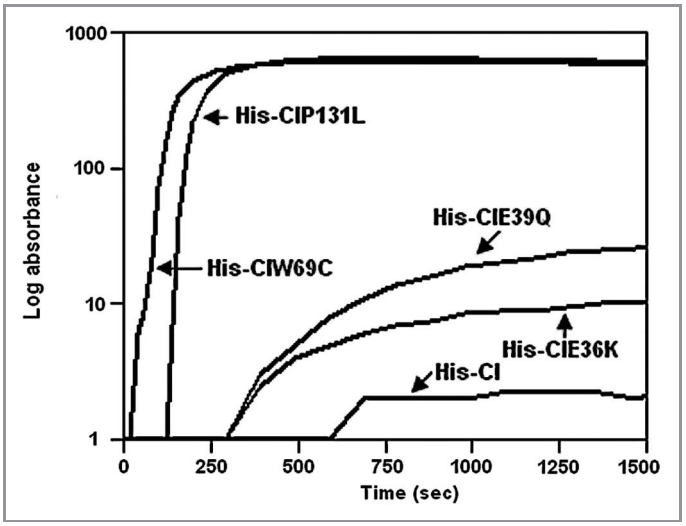

Figure 3. Light scattering of the indicated repressor proteins at 42°C. Absorbance values (as log) of the indicated protein (5 μM) were recorded at 360 nm and plotted against the time of incubation at 42°C.

(**Fig. 5B**). The data are somewhat expected as His-CIW69C possesses six Trp residues, whereas, all other mutants and His-CI carry seven Trp residues.

Stability of the L1 repressor mutants. The intrinsic Trp fluorescence spectra of His-CI and mutant repressors yielded emission maxima at ~335–336 nm at 32°C (data not shown). To obtain clues about the stability of the mutant and the wild-type repressors, we equilibrated all the histidine-tagged repressor proteins with 0–3.5 M GdnCl (guanidine hydrochloride) followed by the recording of their intrinsic tryptophan fluorescence spectra at 32°C. Upon increasing concentrations of GdnCl, the fluorescence intensity values of His-CIW69C were gradually increased, whereas, those of the rest of the proteins were increased gradually after progressive initial decrease (**Figs. 6A–E**). Conversely, the λ_{max} (wavelength of emission maxima) values of all of the proteins were gradually increased when GdnCl concentrations were raised step by step. Roughly monophasic curves were obtained for all repressors when λ_{max} values were plotted against the corresponding GdnCl concentrations (**Fig. 6F**). All of the repressor proteins, however, did not show the unfolding transition at identical GdnCl concentrations. While His-CIW69C showed transition at ~0.25–2 M GdnCl, the rest of the proteins exhibited transitions at ~0.5–2.5 M GdnCl, suggesting that the former repressor starts unfolding at relatively lower GdnCl concentrations. The λ_{max} values at the initiation and at the end points of the transition regions were ~335 and ~350 nm, respectively. There were ~2 nm increase in the λ_{max} value when GdnCl concentrations were raised from ~2 or 2.5 M to 3.5 M. As λ_{max} value of 350 nm or higher indicates the complete exposure of the tryptophan residues to the aqueous solvent,[36] all repressors seemed to be completely unfolded at GdnCl concentrations of ≥ 2 or 2.5 M. To determine the C_m (GdnCl concentrations at the midpoint of unfolding transitions) values of the repressors, the fraction of unfolded repressors were estimated (from the Trp fluorescence data in **Figure 6F** using **Eqn. 2**) and plotted against the respective GdnCl concentrations. From the resulting sigmoidal curves (**Fig. 6F** Inset), the C_m values of His-CI, His-CIP131L, His-CIE36K, His-CIE39Q and His-CIW69C were determined to be 1.41 ± 0.04, 1.40 ± 0.05, 1.31 ± 0.05, 1.39 ± 0.04 and 1.18 ± 0.03, respectively. The C_m values of His-CIW69C and His-CIE46K appeared to be significantly less than those of other repressors (all p values less than 0.05), indicating that these two mutants are the least stable of the five proteins.

Here we have demonstrated that the operator DNA binding activity of CIW69C was completely affected at both 32° and 42°C (**Table 1 and Figure 1**). In contrast, CIP131L weakly resisted the growth of L1cI$^-$ and exhibited reduced operator DNA binding activity at 32°C (**Fig. 1 and Table 1**). Even L1 phage with the P131L mutation in the CI formed lysogen with *M. smegmatis*

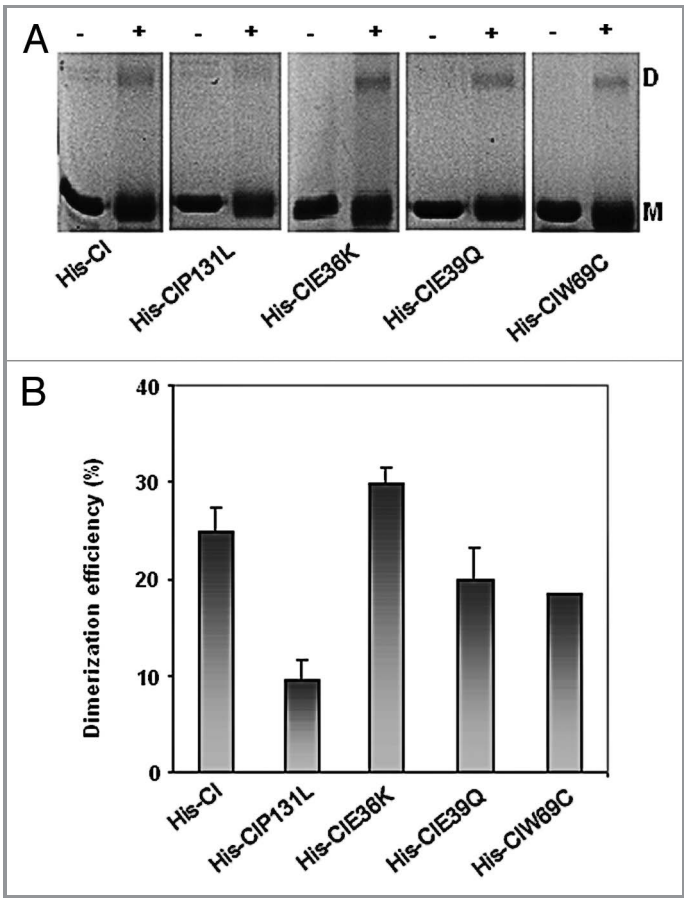

Figure 4. Dimerization ability of the mutant repressor proteins at 32°C. (A) SDS-10% PAGE analysis of the indicated repressor proteins (16 μM each) in the presence (+) and absence (-) of 0.1% gluteraldehyde. D and M denote the dimer- and monomer-specific repressor band. (B) Dimerization efficiency of a repressor protein (in percent) was calculated by dividing the intensity of its dimer-specific band with the cumulative intensities of its dimer- and monomer-specific bands. Prior to estimation, the intensity of the contaminant band (at the dimer-specific band position) in the untreated sample was deducted from that of the corresponding dimer-specific band in the treated sample. Densitometric scanning was done to determine the intensity of each protein band in the gel picture, presented in (A). Error bar indicates the standard deviations from three independent experiments.

at 32°C but not at 42°C.[2] Both mutations, however, made CI thermo-sensitive almost equally at 42°C (**Figs. 2 and 3**). In addition, P131L substitution affected the dimerization (**Fig. 4**), whereas, the W69C change affected both the stability and the tertiary structure of CI at 32°C (**Fig. 5B**). Together the data suggest that the above mutations affected the operator DNA binding activity of CI by distinct mechanism. While the tertiary structure of His-CIW69C may be inappropriate for its binding to O_{64} DNA, inadequate dimerization of His-CIP131L may be involved in its weak operator DNA binding affinity. As L1 repressor was reported to bind to the asymmetric operator DNA as a monomer,[9] the decreased dimerization efficiency of His-CIP131L should not have affected the DNA binding activity of this CTD mutant protein at 32°C. We therefore suggest that alteration of the Pro131 residue somehow partially changed the conformation of DNA binding NTD of His-CIP131L monomer, which in turn led to its reduced binding to the operator DNA at 32°C.

The E36K or the E39Q mutation affected the structure and the dimerization of CI little (**Figs. 2–5**) though both substitutions enhanced the operator DNA binding affinity of CI substantially at 32°C (**Table 1**). Interestingly, the former mutation also made the CI less stable. The increased positive charge in the HTH motif of His-CIE36K may be responsible for its stronger interaction with the O_{64} DNA. In contrast, the decreased negative charge in the HTH motif of His-CIE39Q may reduce the repulsion between this repressor and the O_{64} DNA. In addition, amide side chain of Gln39 residue of His-CIE39Q may form new hydrogen bond(s) with the operator DNA. All these factors together may contribute to the enhanced operator DNA binding activity of His-CIE39Q.

The L1 repressor mutant harboring the temperature-sensitive mutation in the CTD of CI could be exploited in the generation of a mycobateria-specific expression vector only if its operator DNA binding affinity at 32°C can be enhanced by additional mutagenesis. In this respect, clubbing the CIE39Q mutation in the HTH motif with the temperature sensitive mutation in the CTD may yield the double mutant with the better DNA binding ability at 32°C but not at 42°C. Such a double mutant, however, may not be very useful as CIE39Q could not block the growth of L1cI$^-$ completely (**Table 1**). The operator DNA binding affinity of the double mutant, however, could be further increased by introducing additional basic amino acid residue in its HTH motif. The CIW69C and CIE36K mutants may not be useful for the construction of a double mutant as both are relatively unstable at 32°C. Secondly, CIE36K did not show appreciable operator DNA binding activity in vitro. Collectively, the studies on the L1 repressor mutants have provided a solid foundation for generating a repressor mutant that may lead to the creation of a temperature-controlled expression vector.

Materials and Methods

Strains and plasmids. All bacterial and phage strains and plasmids used in the study are listed in **Table S1**. *M. smegmatis* and *E. coli* strains were routinely grown in Middlebrook 7H9[2] and Luria-Bertani[37] media (supplemented with appropriate antibiotic, if required), respectively. Mycobacteriophage L1cI$^-$ and its growth conditions were described previously.[2]

Basic DNA and protein techniques. Plasmid DNA isolation, DNA estimation, digestion of DNA by restriction enzymes, modification of DNA fragments by modifying enzymes, plasmid DNA transformation of *E. coli* or *M. smegmatis*, polymerase chain reaction (PCR), purification of DNA fragments, sequencing of PCR made DNA fragments, labeling of DNA fragments with [^{32}P-γ] ATP (BARC, India), agarose gel electrophoresis, native and SDS-PAGE, urea-PAGE, staining of polyacrylamide gels, etc. were performed by standard procedures[4,7,37,38] or according to the protocols provided by the respective manufacturers (Qiagen; Fermentas GmbH; Bangalore Genei P. Ltd.). The total protein content was estimated by the Bradford assay using bovine serum

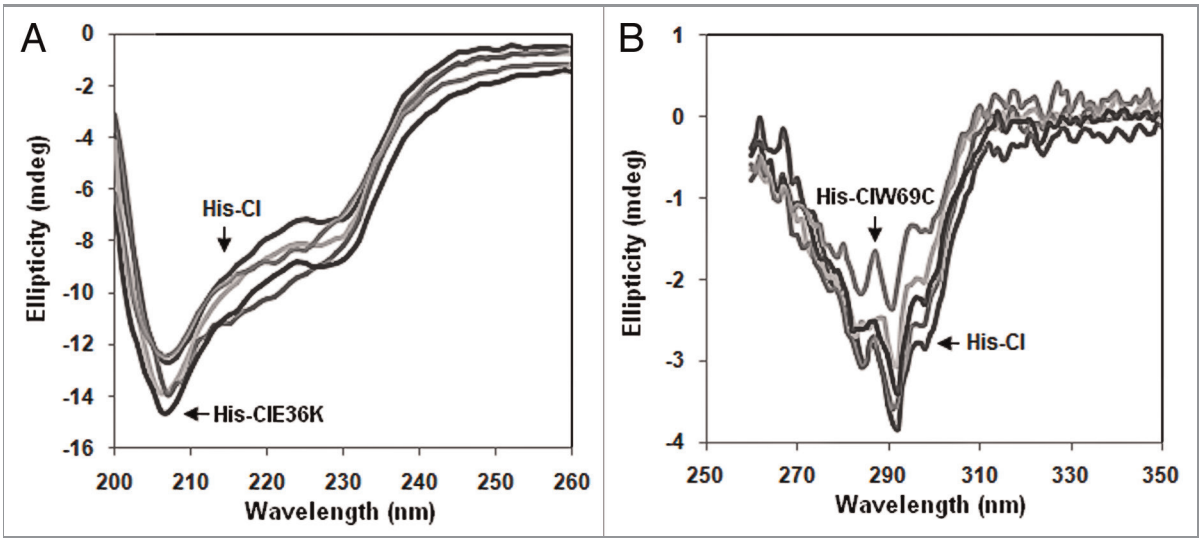

Figure 5. Structure of the repressor proteins. (A) Far-UV and (B) near-UV CD spectra of different mutant repressor proteins at 32°C.

albumin as the standard.[39] Isolation of L1 chromosomal DNA was performed by a previously described procedure.[2]

Plasmid construction. To construct plasmid p1234 (**Table S1**), the ~250 bp XhoI fragment of pSAU1180[7] was replaced by the ~250 bp XhoI fragment of pSAU1085.[3] In the present text, pSAU1085 and pSAU1180 were designated as p1085 and p1180, respectively. The L1 repressor gene in plasmid p1085 harbors a point mutation at the 131st codon that in turn encodes CIP131L, a temperature-sensitive L1 repressor.[3] Plasmid p1234 was used to overexpress the N-terminal histidine-tagged CIP131L (His-CIP131L). For overexpressing other mutant L1 repressors, several overlapping polymerase chain reactions were performed separately by ProofStart Polymerase using specific primers and p1180 DNA as the template. A 610 bp L1 DNA fragment, amplified from p1180 DNA using primer LCP2[7] and a 120 bp L1 DNA fragment [made from p1180 DNA with primers LCP3[7] and LCP6 (5'GCCATACAGCTGTGCGATT)], was cloned into MluN1 digested pCAP[S] (Roche Applied Science) to generate p1263 (**Table S1**). To overexpress His-CIE39Q, plasmid p1264 was constructed by subcloning the ~610 bp EcoRI-HindIII fragment of p1263 into the identical sites of p1232, a pET28a (Novagen, USA) derivative whose BglII site was inactivated by Klenow polymerase treatment. To construct p1262 (**Table S1**), a second 610 bp L1 DNA fragment, PCR generated from the p1180 DNA using primers LCP2 and a DNA fragment [synthesized from p1180 DNA with LCP1[13] and LCP7 (5'CTGCGATTTTGGTCTGAT TAAAC), was cloned into pCR-Blunt II TOPO (Invitrogen). To overexpress His-CIE36K, plasmid p1265 was generated by replacing the 530 bp BglII-HindIII fragment of p1264 with the 530 bp BglII-HindIII fragment of p1262. To overexpress His-CIW69C, p1266 (**Table S1**) was created by cloning an EcoR1 and HindIII digested L1 DNA fragment [PCR made from p1180 DNA with primer LCP2 and a DNA fragment which was amplified using p1180 DNA, LCP3 and PW69C (5'GTGTCCCACGGGCAG TTCT GCTGGACG)] into the identical sites of pET28a. *E. coli* strains S1234, S1264, S1265 and S1266 were constructed by transforming p1234, p1264, p1265 and p1266 to the competent *E. coli* BL21(DE3) cells (Novagen) separately. *E. coli* XL1 Blue or DH5α was utilized as mother to amplify most plasmids described here.

To generate p1268, a ~640 bp L1 DNA fragment [amplified by ProofStart polymerase using primers LCP1 and LCP2 and L1 DNA (as the template)] was cleaved with EcoRI and HindIII together and cloned into the identical sites of pUC19 (**Table S1**). The L1 DNA insert of p1268 did not carry a mutation and bears a ribosome binding site at the upstream of CI encoding ORF. Plasmids p1270, p1272, p1273 and p1276 were constructed by replacing the ~530 bp BglII-HindIII fragment of p1268 with the ~530 bp BglII-HindIII fragments of p1085, p1264, p1265 and p1266, respectively. Finally, p1269, p1271, p1274, p1275 and p1277 were constructed by cloning the EcoRI -HindIII L1 DNA inserts of p1268, p1270, p1272, p1273 and p1276, respectively to EcoRI and HindIII double-digested pMV261[29] (**Table S1**). Plasmid pMV261 and its derivatives were transformed separately into competent *M. smegmatis mc²155* cells according to Sau et al.[3] and the resulting transformants (such as S5036, S5073, S5038, etc.; **Table S1**) were purified, stocked and utilized for investigating the in vivo activities of mutant repressor proteins.

Purification of native and mutant L1 repressor proteins. Each N-terminal histidine-tagged repressor protein was purified from a distinct IPTG-induced *E. coli* cell culture by the same affinity chromatography approach as described previously for the purification of His-CI from S1180.[7] His-CIP131L, His-CIE39Q, His-CIE36K and His-CIW69C were purified from S1234, S1264, S1265 and S1266 cells (**Table S1**), respectively. All mutant L1 repressor proteins were 97–99% pure as estimated from SDS-13.5% PAGE analysis (data not shown). The concentrations of all repressor proteins were calculated using the molecular mass of the respective monomeric repressor.

Limited proteolysis. Nearly 8 μg repressor protein in the phosphate buffer [50 mM phosphate buffer (pH 7.0), 200 mM

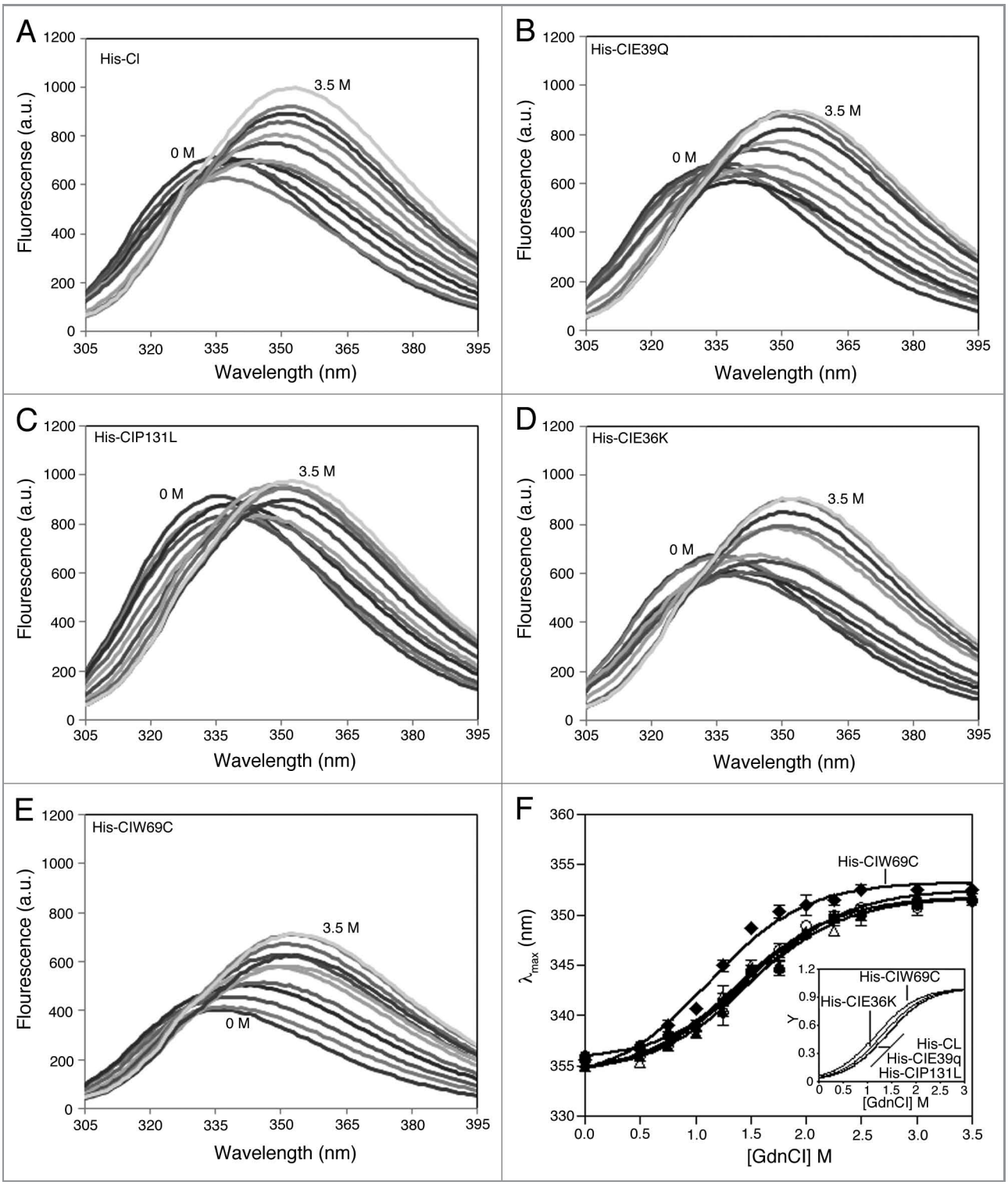

Figure 6. GdnCl-induced equilibrium unfolding of the wild-type and mutant L1 repressor proteins. (A–E) Intrinsic Trp fluorescence spectra of the indicated repressor proteins (each 0.5 μM) in the presence of 0–3.5 M GdnCl. (F) Plots of the λ_{max} values vs. GdnCl concentrations show the equilibrium unfolding of the indicated proteins. The λ_{max} values were derived from (A–E). The lines through the λ_{max} values indicate the best-fit curves. Inset plots show the change in fraction of unfolded protein (*Y*) in the presence and absence of GdnCl. *Y* values were calculated from the λ_{max} values (F) and analyzed by the standard procedure as described in Materials and Methods.

NaCl, 5% glycerol, 1 mM EDTA] was incubated at 32° or 42°C for 20 min followed by its limited proteolysis with 32 ng chymotrypsin according to Ganguly et al.[7]

Circular Dichroism spectra. The far-UV (200–260 nm) and near-UV (250–350 nm) CD (Circular Dichroism) spectra of the 5–30 μM repressor proteins in the phosphate buffer were recorded by Jasco J600 spectrophotometer at 32°C according to the standard procedures.[7,36] The path lengths of the cuvette, used to record the far-UV and near-UV CD spectrum, were 1 mm and 2 mm, respectively.

Chemical cross-linking. Chemical cross-linking of the repressor proteins were performed according to a standard method[7] with minor modification. Briefly, 16 μM repressor protein in 20 μl phosphate buffer solution was treated with 0.1% glutaraldehyde for 2 min at 32°C followed by the analysis of all samples by SDS-10% PAGE.

Thermal aggregation. Temperature-induced aggregation of 5 μM repressor in the phosphate buffer was probed by light scattering at 360 nm in a spectrophotometer (Shimadzu 3000) connected to a 32° or 42°C maintained water bath.[36]

Gel shift assay. To study the equilibrium binding of different repressor proteins to the same ^{32}P labeled O_{64} operator DNA (0.1 nM), separate gel shift assays were performed according to a standard method[7] with modification. Briefly, 20 μl reaction mixture containing a repressor protein and the labeled O_{64} DNA[9] in the phosphate buffer [50 mM Na-phosphate buffer (pH 6.0), 50 mM NaCl, 1 mM EDTA, 5% glycerol and 10 μg/ml bovine serum albumin] was incubated at 32° or 42°C for 20 min followed by the analysis of mixture using native 6% PAGE. Amounts of operator DNA bound by each repressor were determined by scanning the respective autoradiogram. The apparent equilibrium dissociation constant or K_d (repressor concentration yielding half-maximal binding) was determined from the plot of operator DNA bound vs. repressor concentration according to Bandhu et al.[9]

Protein denaturation. To get an idea about the stability of mutant repressors, all histidine-tagged repressor mutants and His-CI (each 5 μM) were equilibrated with 0–3.5 M GdnCl (guanidine hydrochloride) followed by the recording of their intrinsic tryptophan fluorescence spectra (λ_{em} = 300–400 nm, and λ_{ex} = 295 nm) at 32°C with a Hitachi F-3000 spectro-fluorimeter as stated before.[36,40] C_m (GdnCl concentration at the midpoint of unfolding transition) values were estimated by nonlinear fitting of the unfolding data to **Equation 2** using GraphPad Prism (GraphPad Software Inc.) as described.[40]

$$Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{X-Cm}) \quad (1)$$

where X and Y indicate the concentration of GdnCl and the fraction of unfolded protein molecules respectively. Assuming that denaturation of all proteins follows a two-state model,[41] the fraction of unfolded molecules was determined using the following equation:

$$Y = (f_n - f)/(f_n - f_u) \quad (2)$$

where f, f_n and f_u represent the observed emission maxima of protein at any GdnCl concentration, emission maxima of protein at fully native state, and emission maxima of protein at the completely unfolded state, respectively. The last two parameters were determined by a standard procedure as described.[40]

Statistical analysis. All data were presented here as the means of three independent experiments with the standard deviation. To determine mean, standard deviation and p values, data were analyzed by the specific programs of MS Excel. Two results were considered significant if the corresponding p value was less than 0.05.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing interests.

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Supplemental Material

Supplemental material may be downloaded here:
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