Promoter Activation by CII, a Potent Transcriptional Activator from Bacteriophage 186*

Iain Murchland, Alexandra Ahlgren-Berg, David G. Priest, Ian B. Dodd, and Keith E. Shearwin

From the Department of Biochemistry, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

The lysogeny promoting protein CII from bacteriophage 186 is a potent transcriptional activator, capable of mediating at least a 400-fold increase in transcription over basal activity. Despite being functionally similar to its counterpart in phage λ, it shows no homology at the level of protein sequence and does not belong to any known family of transcriptional activators. It also has the unusual property of binding DNA half-sites that are separated by 20 base pairs, center to center. Here we investigate the structural and functional properties of CII using a combination of genetics, in vitro assays, and mutational analysis. We find that 186 CII possesses two functional domains, with an independent activation epitope in each. 186 CII owes its potent activity to activation mechanisms that are dependent on both the α70 and α C-terminal domain (αCTD) components of RNA polymerase, contacting different functional domains. We also present evidence that like λ CII, 186 CII is proteolytically degraded in vivo, but unlike λ CII, 186 CII proteolysis results in a specific, transcriptionally inactive, degradation product with altered self-association properties.

Temperate bacteriophage have proven to be fruitful systems for the study of prokaryotic transcription, gene regulatory networks, and biological decision making. Many seminal contributions to these fields have been based on studies using the model system of the λ coliphage (1). However, investigation of λ only gives us insight into one instance of a solution to the various biological problems that a bacteriophage must solve. One way of working toward an understanding of the general principles governing these problems and their solutions is through the study of functionally similar, yet evolutionarily divergent systems.

One such system is the temperate coliphage 186, a member of the P2-like family, which has a similar network topology to that of λ, but no detectable sequence homology (2). Here we examine the CII protein of 186, which like λ CII, is a potent transcriptional activator and is essential for the establishment of the lysogenic life cycle of the phage (3–5). 186 CII promotes establishment of lysogeny by activation of the pE promoter, which is one of two promoters that drive expression of the maintenance repressor CI (Fig. 1). Activation of pE is dependent only on CII and RNAP, and is necessary for establishment of lysogeny, but not its maintenance (6). CII protein itself is produced upon infection via the early lytic transcript under the control of pr. This results in an apparent paradox in which expression of the lytic transcript promotes lysogeny via the activity of CII. The switch region of λ yields a similar situation, which is resolved by the fact that λ CII is rapidly degraded by the host protease FtsH, such that λ CII only accumulates to the level necessary for establishment of lysogeny in a small fraction of infections (7–10). Whether proteolysis of 186 CII plays a similar role in the life cycle decision of bacteriophage 186 remains an open question.

The CII protein is 169 amino acids in length, with a molecular mass of 18.7 kDa and a predicted helix-turn-helix motif in the N-terminal region (4, 11). The helix-turn-helix motif is presumed to be responsible for binding of CII to two inverted repeat 7-mer half-sites of the sequence ATGTTTG. 186 CII binds as a pre-formed dimer to the half-sites, the centers of which are 20 bp apart (Fig. 1) and upon binding induces moderate DNA bending of 40–45° (6). Binding of CII and its ability to activate the pE promoter is sensitive both to small changes in the spacing of the half-sites as well as larger changes in spacing, which preserve helical phasing, suggesting that the geometry of the dimer is relatively rigid (6).

The unusual arrangement of the CII binding sites at pE is not only distinct from that of λ CII, which recognizes direct repeats in close proximity to each other, but is highly unusual in the broader context of prokaryotic transcriptional activators. Few examples of activators with similarly wide spacing between half-sites are known, which poses a question as to whether CII activates transcription via novel mechanisms. The mechanism of CII activity is made even more interesting by its potency; 186 CII is capable of ~400-fold activation of pE (4). Previous work has shown that 186 CII acts via recruitment of RNAP to the promoter, but the way in which this is achieved remains unknown (6).

* This work was supported by Australian Research Council Grants DP0665185 and DP110100824.

1 To whom correspondence should be addressed. Tel.: 61-8-8313-5361; Fax: 61-8-8313-4362; E-mail: keith.shearwin@adelaide.edu.au.
Here we demonstrate that unlike λ CIIR, the CIIR protein of 186 is rapidly degraded in vivo. We investigate the domain organization and the transcriptional activation mechanism of CIIR using genetic screens to isolate various classes of mutations, and then characterizing how these mutations exert their effects using genetic and biochemical methods. We find that proteolytic degradation of CIIR compromises the multimerization and thus indirectly the DNA binding of CIIR by removing part of its C-terminal domain. Here we demonstrate that like CIIR, the CIIR protein of 186 is rapidly degraded in vivo. We investigate the domain organization and the transcriptional activation mechanism of CIIR using genetic screens to isolate various classes of mutations, and then characterizing how these mutations exert their effects using genetic and biochemical methods. We find that proteolytic degradation of CIIR compromises the multimerization and thus indirectly the DNA binding of CIIR by removing part of its C-terminal domain. Here we demonstrate that like CIIR, the CIIR protein of 186 is rapidly degraded in vivo. We investigate the domain organization and the transcriptional activation mechanism of CIIR using genetic screens to isolate various classes of mutations, and then characterizing how these mutations exert their effects using genetic and biochemical methods. We find that proteolytic degradation of CIIR compromises the multimerization and thus indirectly the DNA binding of CIIR by removing part of its C-terminal domain. Here we demonstrate that like CIIR, the CIIR protein of 186 is rapidly degraded in vivo. We investigate the domain organization and the transcriptional activation mechanism of CIIR using genetic screens to isolate various classes of mutations, and then characterizing how these mutations exert their effects using genetic and biochemical methods. We find that proteolytic degradation of CIIR compromises the multimerization and thus indirectly the DNA binding of CIIR by removing part of its C-terminal domain.

EXPERIMENTAL PROCEDURES

Given the prevalence and importance of various truncations of CIIR in this study, for the sake of clarity we will hereafter refer to wild-type CIIR as CIIR169, denoting its length of 169 amino acids, and refer to other truncations using the same convention.

Oligonucleotides—A summary of all oligonucleotides used in this study is provided in Table 1. Oligonucleotides were purchased from Geneworks (Adelaide, Australia).

Plasmids and Bacterial Strains—A summary of all plasmids (12) used in this study is provided in Table 2. Reporters were constructed using a plasmid integration system developed from the CRIM plasmids (13, 14), incorporating promoter fragments between KpnI and XbaI restriction sites, followed by an Rnas3II cleavage site (15) and an lacO2 lacZ reporter gene (16). The pE (–120 to +115) reporter fragment was PCR amplified

2 The abbreviations used are: RNAP, RNA polymerase; αCTD, C-terminal domain of the α subunit; IPTG, isopropyl β-D-galactopyranoside; SEC-MALS, size-exclusion chromatography coupled with multiangle light scattering; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NTD, N-terminal domain.
NdeI sites, and the 186 CII residues 83–192 between the NdeI/ sites, the pET RBS from pZS45-CII169 between the HindIII/ sites. The pET15b vector (Novagen). Plasmid pZE1-CII169 was constructed by ligating the NdeI/BamHI insert of pZS45 into the pET15b vector. The pZE15 vector system (17), with the small XhoI/AvrII hexamer in underline, all derivatives of Escherichia coli mutagenesis of pHTf1-rpoA with primers 1239 and 1240. Introduction of a stop codon after residue 235 by QuikChange site-directed mutagenesis of pHTf1-rpoA with primers 1239 and 1240. The pITCH-pCIIR-LacZ was integrated pE promoter fragment and cloning strategy was used to construct the matching pBC1-pE reporter construct for constitutive expression of LacI from the wild-type ORF and promoter. Western Blots— BW25113 or PN376 cells harboring pZS45-CII169 or pZS45-CII145 by high fidelity PCR with primers 289 and RSP (see Table 1 for sequences). The resulting PCR product was used as template for error-prone PCR with Tag DNA polymerase (New England Biolabs) and MgCl2 supplemented to a total concentration of 6 mM. The product of error-prone PCR was subcloned back into pZS45-CII145 via BamHI and Xhol restriction sites and transformed into screening strain IM18 by electroporation. Transformants were plated onto TB agar supplemented with 100 µg ml−1 of ampicillin, 50 µg ml−1 of kanamycin, 50 µg ml−1 of spectinomycin, 40 µg ml−1 of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), 200 µM IPTG and incubated at 34 °C for 24 h, followed by 4 °C for 2–3 days to allow color development, before selection of healthy white colonies. LacZ Assays—Kinetic LacZ assays in a 96-well microtiter plate were performed as previously described (20). Western Blots—BW25113 or PN376 cells harboring pZS45-CII169 or pZS45-CII145 were grown in LB supplemented with 100 µM IPTG at 37 °C to mid-log phase. Cells were lysed using B-PER (Pierce) and Benzonase (0.25 units µl−1) (Novagen) and cell extracts were run on NuPAGE BisTris (12 or 4–12%) or

**TABLE 2**

Plasmids used in this study

| Plasmid name            | Description                                                                 | Features                                      | Reference |
|-------------------------|-----------------------------------------------------------------------------|-----------------------------------------------|-----------|
| pET15b-CII169, pET15b-CII145, pET15b-CII135 | Vectors for IPTG-inducible over-expression of His₆, (thrombin cleavable) tagged CII and derivatives. Source of purified CII used in this study. | ColE1 ori; ampicillin resistance              | This work |
| pZS45                   | Multipurpose, modular, low copy vector                                       | SC101 ori; spectinomycin resistance           | 17 and 18 this work |
| pZS45-CII169, pZS45-CII145, pZS45-CII135 | Low copy vectors for expression of CII and derivatives under inducible control of Lac promoter | SC101 ori; spectinomycin resistance           | This work |
| pZE1-pE-CI-CTD          | pZE15 (18) derivative high copy vector for CII-inducible expression of 186 CII C-terminal domain (residues 83–192) with thrombin-cleavable 6×His tag | pBR322 ori; ampicillin resistance             | 12        |
| pHfH1-rpoA              | Vector for inducible expression of RNAS α subunit (and derivatives) under control of LacI repressed promoter | pBR322 ori; ampicillin resistance             | 41        |
| pVRr                    | Vector for constitutive expression of α subunit (and derivatives)            | pBR322 ori; ampicillin resistance             | 23        |
| pHfH7f1-HNhα, pHfH7f1-HNhα(1–235), pHfH7f1-α, pMK5e2, pT7β | Vectors for over-expression of RNAS subunits Source of reconstituted RNAS used in this study pβCI (24) derivative encoding pE (−120 to +115) flanked by strong termination signals for in vitro transcription experiments | ColE1 ori; ampicillin resistance              | This work |
| pHfH8-CI-Pl             | For constitutive expression of LacI from the wild-type ORF and promoter      | pLa5a ori; kanamycin resistance               | H. Bujard, Heidelberg University, Germany |
| pITCH-pCIIR-LacZ        | pCIIR lacZ reporter construct for chromosomial integration at λ attB        | R6K-γ ori; chloramphenicol resistance         | This work |
| pITCH-pCIIR-LacZ        | pCIIR lacZ reporter construct for chromosomal integration at HK022 attB    | R6K-γ ori; chloramphenicol resistance         | This work |

The target plasmid pZS45-CII169 was mutagenized using the GPS-LS transposon-based linker scanning mutagenesis kit (New England Biolabs). The terminated transposition reaction was transformed into electrocomptent DH5α cells and plated and onto selective media. Insertions were identified by colony PCR using the T7prom primer, with the supplied N and S transprimers. The protocol was completed by Pmel digest and re-ligation of plasmids carrying insertions, resulting in a 15-bp scar at the insertion site. Truncations generated by this method encode a C-terminal valine residue following the last wild-type CII residue.

**Genetic Screen for CII Activation Mutants**—CII145 was amplified from pZS45-CII145 by high fidelity PCR using primers 289 and RSP (see Table 1 for sequences). The resulting PCR product was used as template for error-prone PCR with Taq DNA polymerase (New England Biolabs) and MgCl2 supplemented to a total concentration of 6 mM. The product of error-prone PCR was subcloned back into pZS45-CII145 via BamHI and XhoI restriction sites and transformed into screening strain IM18 by electroporation. Transformants were plated onto TB agar supplemented with 100 µg ml−1 of ampicillin, 50 µg ml−1 of kanamycin, 50 µg ml−1 of spectinomycin, 40 µg ml−1 of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), 200 µM IPTG and incubated at 34 °C for 24 h, followed by 4 °C for 2–3 days to allow color development, before selection of healthy white colonies.
Novex 12.5% Tricine gels (Invitrogen) in 1 \times \text{NuPAGE MES SDS Running Buffer} or Novex Tricine SDS Running Buffer, respectively (Invitrogen). Gels were blotted using an iBlot and Gel Transfer Stacks PVDF (Invitrogen) or the Novex wet transfer apparatus onto Hybond-LFP PVDF membrane (GE Healthcare). Membranes were blocked using 5% BSA. CII and A1 primary detection was with rabbit antisera (IMVS Veterinary Services). Secondary detection used goat anti-rabbit IgG Cy5-labeled ECL plex secondary antibody (GE Healthcare). Membranes were scanned using a Typhoon Trio (GE Healthcare) and images analyzed by ImageQuant (GE Healthcare).

**Half-life Determinations—**BW25113 cells harboring pZS45-CII169 or pZS45-CII145 were grown in LB supplemented with 50 mM spectinomycin and 100 \mu M IPTG at 37 °C to mid-log phase. Ten minutes prior to the assay start 50 mM heparin, 0.2 mM rATP, rCTP, and rGTP each, 0.02 mM rUTP, 250 mM imidazole, 500 mM NaCl (TEG100) was employed. CII-containing protein was separated by SDS-PAGE, the region around 15.5 kDa was excised, washed in 500 \mu M of 100 mM ammonium bicarbonate, destained, reduced with dithiothreitol, and alkylated with iodoacetamide before being treated overnight with 200 ng of Asp-N (Promega, catalog number V1621) in 10 mM ammonium bicarbonate containing 10% acetonitrile. The reaction was halted with 30 \mu M of 1% formic acid, sonicated for 15 min, and buffer was removed. Two further trifluoroacetic acid/acetoniitride extractions were performed. Extracts were pooled and separated on a HPLC system (Thermo Scientific) using a separation column (Thermo Scientific) (Acclaim PepMap RSLC, 75 \mu m diameter \times 15 cm length) and a trapping column (Thermo Scientific) (Acclaim PepMap100, 75 \mu m diameter \times 2 cm length). The HPLC system was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The 6 most intense peptide ions from each scan with charge states \geq 2 and minimum signal intensity of 1000 were sequentially isolated and fragmented in the high-pressure linear ion trap by low-energy CID.

**SEC-MALS—**Size exclusion chromatography was carried out using a SuperdexTM 200 10/300 GL column (GE Healthcare) eluted in PBS. MALS analysis used in-line detection with Wyatt MiniDawn Treos and Opti-lab rEx instruments, and analysis was conducted using Astra version 5 software (Wyatt Technology). Samples were 2 mg/ml, using injection volumes of 100–300 \mu l. Molecular Modeling—Structure predictions for the N-terminal domain generated 48,000 decoys using the abrelax protocol of Rosetta 3.1 (26) followed by clustering of the 500 lowest energy structures to identify favorable models and assess convergence. Docking of the N-terminal domain to DNA used the HADDOCK webserver (27, 28). UCSF Chimera (29) was used for structure depiction, analysis, and preparation of figures.
Promoter Activation by 186 CII

RESULTS

Domain Organization of CII—We used transposon-mediated linker insertion mutagenesis to investigate the functional organization of the CII protein. Linker insertion mutagenesis creates 15-bp insertions at random locations within the DNA sequence of interest, in this case a plasmid (pZS45-CII169) encoding CII169. Due to the sequence of this insert and the fact that it inserts independently of the reading frame, it can give rise to mutants possessing either a 5-amino acid translation insertion (“insertion mutants”), or a premature stop codon (“truncation mutants”). Insertion mutations in regions of structural or functional importance (such as solvent-excluded sites, sites of intermolecular interaction, and some regions of secondary structure) will result in substantially reduced function of the protein. In contrast, insertion mutations that retain substantial activity can be expected to lie in solvent-exposed regions, without direct functional importance or significant secondary structure. Thus previous work using similar techniques have shown that the activity of insertion mutants is correlated with insertions that lie within inter-domain linkers, or solvent-exposed loops (30–33).

We isolated and sequenced 26 independent mutants via this method, comprising 10 truncation mutants, and 16 insertion mutants (Fig. 2A). Only four of these mutants retained at least 50% wild-type activity, assayed using a pE lacZ in vivo reporter. One of these encoded an insertion after residue Leu-87, whereas two independent insertions after nearby residue Cys-81 retain moderate activity, suggesting that this region is an inter-domain linker. This is in accordance with bioinformatic predictions that CII169 is composed of two structural domains with a boundary near residue Cys-81 using the Ginzu algorithm (34).

The other high activity mutants lie near the C terminus of the protein, insertions after Ser-153 and Phe-166, and a truncation following Ser-160. It is perhaps unsurprising that these mutations suggest that this C-terminal region is unimportant for structure and function, and can be discarded or disrupted without losing function. Unexpectedly, however, two of these were gain of function mutations, causing higher expression of the pE reporter. Observing that loss or interruption of C-terminal residues increased CII in vivo activity, we assayed the activity of a series of truncation mutants between 141 and 166 amino acids in length (Fig. 2B). Higher activity was correlated with shorter CII protein length, until activity dropped sharply with truncation to 141 amino acids, likely due to loss of structure and function of the C-terminal domain.

We also observed that truncations on the C-terminal side of the putative domain boundary around amino acid 81 retained low level activity (5–10% of wild-type), whereas those lying on the N-terminal side were inactive (<2% of wild-type activity) (Fig. 2C). These observations are consistent with the prediction of an N-terminal helix-turn-helix DNA binding domain (4, 11). The low activity of C-terminal truncations at or before residue 141 could indicate a loss of an independent DNA-binding function, loss of transcriptional activation, or a loss of self-association, leading to reduced DNA binding due to loss of cooperativity.

CII169 Is Rapidly Degraded in Vivo—Given its functional similarity to λ CI, we hypothesized that 186 CII may also be proteolytically degraded in E. coli. The C terminally truncated gain of function mutations (Fig. 2, B and C) could then be explained by loss or disruption of the proteolysis signal. Examining the in vivo stability of CII169 by Western blot following addition of a translation inhibitor reveals a calculated half-life of 2.6 min (2.3 ≤ t_1/2 ≤ 3.0 min, 95% CI) (Fig. 3A), which is similar to the half-life of λ CI (7, 8, 35). Western blot analysis also revealed that a specific degradation product is evident for CII169 (Fig. 3A, inset), which is suggestive of proteolytic degradation of CII169 by a site-specific protease.

Consistent with a loss of proteolytic degradation in the CII145 mutant, we observed higher in vivo activity, and saturation of activity at lower levels of induction of CII145, compared with CII169 (Fig. 3B). Indeed, CII145 has a measured half-life of −38 min (31 ≤ t_1/2 ≤ 49 min, 95% CI) (Fig. 3A), shows higher equilibrium cellular concentrations as detected by Western blot, and shows no evidence of any specific degradation product (Fig. 3A, inset). In vitro transcription assays using purified CII145 and CII169 with N-terminal His tags show no statistically significant difference in the specific activities of the two proteins (Fig. 3C), demonstrating that higher in vivo activity is due only to increased cellular concentrations, not a change in the way CII145 activates transcription.

We reasoned that the C-terminal portion of CII169 is likely responsible for recruitment of a protease. To test our hypothesis, we created a translational fusion of the proteolytically stable protein λ CI (36), with 186 CII residues 146–169 (ACI-fusion), and compared its stability to wild-type λ CI through translation stop experiments and Western blot analysis probing for λ CI (Fig. 3D). As expected, no degradation of wild-type λ CI is evident within the duration of the experiment (29 min). In contrast, the λ CI fusion protein is substantially degraded within 15 min, and almost completely depleted by 29 min. Thus we conclude that the last 24 amino acids of 186 CII169 constitute a degradation signal, which is sufficient to destabilize an otherwise stable protein. Interestingly, no specific degradation product is evident for the λ CI fusion protein.

A Genetic Screen for Less Active CII145 Mutants That Retain DNA Binding—To complement our structural insights, we set out to characterize which regions of 186 CII are responsible for its activity, and investigate the mechanism of transcriptional activation by CII via a mutational screen for loss of function (Fig. 4A). To avoid confounding our analysis with gain of function mutations that increase CII169 stability, we conducted our genetic screen using the stabilized CII145. This approach also allowed higher cellular concentrations to be achieved, leading to a greater measurement window in our selection assays. Because the specific activities of CII145 and CII169 are equal (Fig. 3C), we could be confident that the activation mechanisms are shared between the two forms. A simple genetic screen for mutations that abrogate CII transcriptional activity would yield many mutations that cause loss of function via loss of structure and/or DNA binding, rather than loss of transcriptional activation. We reasoned that a dual selection screen, in which we select for loss of pE transcriptional activity, but against loss of DNA binding, would yield greater insight.
To achieve this, we designed a synthetic promoter in which two pairs of CII recognition half-sites straddled the $-10$ and $-35$ $\sigma^{70}$ binding sites, anticipating that binding of CII at this promoter would compete with RNA polymerase and cause repression of promoter activity (Fig. 4B). Degenerate bases in the $-35$ site generated a library of promoters with varying activities. Repression of the promoter should occur by competition, so we preferred promoters with a weaker affinity for $\sigma^{70}$, expecting they would be more effectively repressed. The basal activities of 52 members of the library were assayed on a multiple copy plasmid. Of these, the 7 promoters with lowest activity were integrated into the host chromosome in single copy and assayed in the presence of IPTG-inducible CII169, supplied from pZS45-CII169 to test for repression by CII169. All 7 promoters demonstrated dose-dependent repression by CII169 (Fig. 4B). A weak negative correlation was observed between the extent of repression by CII169 and the basal activity of the promoters, consistent with our expectations. Promoter clone 19 (hereafter referred to as pCIIR) was selected for use in our genetic screen and sequenced (see “Experimental Procedures” for full sequence). Our assays show that pCIIR was repressed by almost 80% by CII145, but only around 25% by CII169, indicat-
Promoter Activation by 186 CII

FIGURE 3. Comparison of the degradation and activities of CII169 and CII145. A, in vivo degradation of CII169 and CII145. Data points represent individual background-subtracted measurements of cellular CII concentration by Western blot from two independent experiments (a representative example is shown inset; contrast/brightness have been adjusted for presentational purposes). Measurements were normalized to their respective t0 samples. Lines represent fitting of a one-phase exponential decay, assuming decay from 100 to a plateau of 0 \( (R^2 = 0.974 \text{ for CII169}; R^2 = 0.891 \text{ for CII145}) \). B, CII145 exhibits higher in vivo pE activity than CII169. LacZ reporter assays were conducted using strain IM26, and CII variants were expressed from pZS45-based plasmids. The plot shows mean and 95% confidence interval (\( n = 6 \)) and fitted Hill curves for CII169 and CII145. C, CII169 and CII145 have the same activity in vitro. Data points represent independent experiments, measured by phosphorimage analysis of the pE band on polyacrylamide, 8 M urea gels (see inset for an example of the CII concentration range 320 pm to 1 \( \mu \)g images are composites of regions from the same gel with identical brightness/contrast adjustment). The RNAI band in each lane serves as an internal CII-independent control. Band densities were normalized by calculation of the pE:RNAI band ratio, subtraction of background, and calculation as a percentage of the 200 nm CII145 lane on the respective gel. D, CII residues 146–169 destabilize \( \lambda \) CI. Translation stop time course and Western blot detection show that \( \lambda \) CI alone is not detectably degraded, whereas the translational \( \lambda \) CI fusion (186 CII 146–169) is degraded. Time measurements represent the time interval since the reference measurement was taken.

ing a substantial measurement window for detection of mutants with compromised DNA binding (Fig. 4C).

Selection for reduced pE activity was achieved by constructing a gene circuit in which strong transcription from pE brings about host cell death via induction of the lytic life cycle of a 186 cII\( ^a \) prophage. At permissive temperatures (\( \approx 34 ^\circ \text{C} \)) the lysogen is maintained by the CII\( ^a \) repressor. If induced by increased temperature or other means, the lytic phase of the prophage is induced, causing host cell death or severe growth impairment. Previous work (37, 38) has shown that expression of the 186 CII C-terminal domain (CII-CTD) to titrate away functional CII\( ^a \) is critical for survival. These observations are consistent with the view that CII-CTD maintains CII cytoplasmic activity via an intramolecular interaction with the CII-CTD (39). A previous study (40) provided evidence for a CII-CTD-mediated reduction in CII cytoplasmic activity, which was not observed in the current investigation.

Selection for activation mutants was combined with our synthetic pCIIR lacZ reporter construct. Thus, isolation of surviving colonies exhibiting low \( \beta \)-galactosidase activity in a strain with a chromosomal copy of pCIIR driving expression of lacZ enabled screening for CII145 mutants with low transcriptional activity but near wild-type DNA binding.

From three independent rounds of error-prone PCR mutagenesis and selection, 73 isolates of \( \approx 1000 \) colonies were identified for more detailed analysis. The CII-encoding plasmids were purified from these isolates and transformed into two separate reporter strains (JM13 and JM26) for quantitative analysis of pE and pCIIR activities, respectively, by LacZ assay. Isolates exhibiting pCIIR activities less than 8.5 units at 0.2 mM IPTG were sequenced, yielding 12 unique mutations at 7 different amino acid positions (Fig. 4D). The chosen cut-off corresponds to 1.65 S.D. above the mean of pCIIR activity for wild-type CII145, and 95% confidence that repression is equal to or better than wild-type. The mutations form two clusters; one in the N-terminal DNA-binding region, and a second in the C-terminal region (Fig. 4E). Mutants at two positions in particular, Glu-46 and Arg-115, were isolated repeatedly and from independent mutagenesis reactions, suggesting that these may be critical residues from the two clusters. Examining this possibility, we used site-directed mutagenesis to construct the charge reversal R115E mutant, which was shown to have a greater defect in activation than other substitutions at this position (Fig. 4D). We combined R115E with mutations R17L, E46G, or E46K in the N-terminal domain cluster that had a large effect on pE activity. Fig. 4D summarizes pE and pCIIR activities for all mutants, both random and site-directed. Notably, combining the R115E and E46K mutations to create a CII145\( ^{146E/115R} \) double mutant reduced activity on the pE reporter ~4-fold, relative to the E46K single mutant (\( p < 0.05 \); unpaired \( t \) test).
The CII145E46K/R115E exhibits no statistically significant activity on the pE reporter relative to an empty vector control, without significantly compromising DNA binding as assessed by pCIIR activity. Thus, we can conclude that these two mutations are sufficient to disrupt any and all mechanisms by which CII activates transcription.

Mechanisms of CII Activation of pE—Having established that Glu-46 and Arg-115 are critical to transcriptional activation by CII145, we set out to determine how these residues contribute to the function of CII145. Due to the proximity of the CII binding site to the expected location of the pE − 35 promoter element (Fig. 1), we hypothesized that CII145 contacts the σ70
Promoter Activation by 186 CII

subunit of RNA polymerase. Based on the mechanisms by which other factors promote transcription, we also hypothesized that CII145 may contact the aCTD of RNA polymerase. Mutants E46K and R115E were selected for this investigation on the basis that they exhibit the greatest defects at the two critical sites. The strong R17L mutant was also included because it lies some distance in the primary sequence from the key N-terminal site Glu-46, and so could form part of either the Arg-115 or Glu-46 epitope dependent upon the tertiary structure of CII.

CII Activation via RNAP a Subunit—Potential contacts between CII and aCTD were investigated by expression of full-length a (a329) or a235, a truncation of a at residue 235, which deletes the aCTD. It is important to note that because the chromosomal copy of the rpoA gene encoding the a subunit is essential, wild-type a is expressed in addition to the plasmid-encoded variant of a in all assays. With this experimental design, the pool of RNAPs in the cell comprises a mixture of those with 0, one or two plasmid-derived a subunits. Nonetheless, if expression of a235 reduces transcriptional activation relative to an a329 control, this suggests that aCTD is involved in activation at that promoter, making it a simple, useful screen for evidence of aCTD involvement.

Such a decrease in pE activity is evident for CII145 mutants R17L and E46K, but not R115E or wild-type CII145 (Fig. 5A), strongly suggesting that aCTD contributes to activation by the R17L and E46K mutants. No statistically significant decrease is evident for wild-type or R115E CII145, meaning that R17L and E46K mutants. No statistically significant decrease is evident for wild-type or R115E CII145, meaning that aCTD contributes to activation by the R17L and E46K mutants. No statistically significant decrease is evident for wild-type or R115E CII145, meaning that aCTD may or may not contribute to the activities of these variants, because we do not know the magnitude of the effect we should see in the context of potential redundancy of activation mechanisms, and the confounding effects of the chromosomal (wild-type) copy of rpoA. Given the role of aCTD in activation by the R17L and E46K mutations, it seems unlikely that aCTD is not involved in activation by wild-type CII145. However, the results offer the possibility that the R115E mutation abrogates the role of aCTD, warranting further investigation.

To examine the role of aCTD in wild-type and R115E CII145 more thoroughly, we used in vitro transcription (Fig. 5B). pBC2-pE was supplied as supercoiled template for in vitro transcription using either wild-type or R115E CII145, and RNA polymerase reconstituted with either a329 or a235. The plasmid template produces multiple transcripts, including one from pE as well as the RNA1 transcript, which is independent of CII and serves an internal control. This analysis shows that transcription from pE using RNAP incorporating a329 is considerably lower in the presence of CII145 R115E than wild-type CII145 (Fig. 5B, lanes 3 and 5), consistent with the in vivo data. Importantly, however, there is no discernable difference in transcription between wild-type and R115E CII145 variants when transcription is driven by a235-containing RNAP (Fig. 5B, lanes 4 and 6). We also observe that although deletion of the aCTD results in a large decrease in transcription in the presence of wild-type CII145 (Fig. 5B, lanes 3 and 4), there is no similar reduction in transcription in the presence of R115E CII145 (Fig. 5B, lanes 5 and 6). This shows that aCTD-dependent activation of pE is completely lost in the R115E mutant. We conclude that CII145 activates transcription via the aCTD, likely due to a direct contact between aCTD and CII145. We cannot formally exclude the possibility that rather than disrupting a direct contact, the R115E mutation indirectly inhibits the role of the aCTD by, for instance, altering the CII145 structure or the conformation of the promoter DNA. However, our find-
ing that CII145 R115E does not significantly alter DNA binding, as measured by pCIIR activity (Fig. 4D), suggests that substantial changes to the CII145 structure and DNA binding are unlikely. In addition, our in vitro transcription experiments demonstrate that CII145 possesses a second, αCTD-independent mechanism, as both wild-type and R115E CII145 retain partial activity in assays using RNAP that lacks any αCTD (Fig. 5B, lanes 4 and 6).

The Role of σ70 in CII Activation—We used genetic analyses to investigate the possibility of contact between CII and σ70. We looked for contacts with domain 4 of σ70, specifically residues 591–600, which have previously been shown to contact other transcriptional activators (39–41). For each selected mutant of CII, we expressed σ70 mutants of charged residues in this region and analyzed pE activity, looking for reduced wild-type CII145 activity, and genetic suppression of the transcriptional defect of our CII145 mutants (Fig. 5C). None of the tested σ70 variants resulted in a clear reduction in reporter expression due to CII145. However, the K593A variant of σ70 partially suppresses the activation defects of the R17L and E46K mutants, but not the R115E mutant of CII145 (Fig. 5C). In light of this result, we also tested the charge reversal K593E σ70 mutant. Expression of K593E σ70 yielded almost complete restoration of the activity of E46K and R17L CII145, but not the R115E variant (Fig. 5C). Genetic suppression of the R17L and E46K defects by changes in σ70 suggest that CII145 activates via a σ70-dependent mechanism, and that Lys-593 or nearby residues are important to that mechanism. However, it must be noted that as a corollary we would normally expect, but do not observe, a reduction in wild-type or R115E activity associated with the expression of our CII145 mutants (Fig. 5C). This discrepancy could be explained either by some form of redundancy in the mechanism, or by the mechanism relying on σ70 residues that were not investigated here (see "Discussion" for a more detailed examination).

Identification of the CII169 Cleavage Product—To further characterize the structure and function of CII, we sought to identify the sequence of the CII169 proteolytic cleavage product. We have observed that over-expression of CII169 results in further accumulation of full-length protein, but not the degradation product, presumably due to saturation of the responsible protease (8). Thus, isolating the degradation product for direct characterization was problematic. Nonetheless, we were able to employ two approaches to this problem. Initially, we used Western blot analysis of our linker insertion mutants to deduce the location of proteolytic cleavage. CII169 variants containing a 5-amino acid insertion exhibit a higher apparent molecular weight after SDS-PAGE and Western blot analysis. Whether the same is true or not of the degradation product allows us to deduce whether the insertion lies within the degradation product, or is removed by proteolysis (Fig. 6A). We observe that insertions near the N terminus, such as that after residue 4 (4ins) are evident in the degradation product, whereas the converse is true of an insertion near the C terminus after residue 166 (166ins), demonstrating that proteolysis is occurring at the C-terminal end of CII169. The presence of the insertion in the degradation product is evident up to and including an insertion after residue 131, allowing us to conclude that the degradation product comprises at least the first 132 residues of CII169. We also observe that the degradation product has an apparent molecular weight lower than that of the CII142 truncation mutant. Thus we deduce that the C-terminal end of the degradation product lies between residues 132 and 142.

To locate the proteolytic cleavage site more precisely, we used mass spectrometry. Briefly, CII169 was expressed from the low copy number plasmid pZS45-CII169, enriched by heparin affinity chromatography (4), and separated by SDS-PAGE. A faint band as visualized by Coomassie stain was observed migrating at a position consistent with the degradation product, which was excised and treated with Asp-N protease. The resulting peptide fragments were identified by LC-MS/MS (Fig. 6B). 63% sequence coverage was achieved. Asp-N protease has a high specificity for cleavage on the N-terminal side of aspartate and, at a lower rate, glutamate residues (42). In vivo proteolysis of CII169 is expected to yield peptide fragments with C-terminal sequences that do not conform to the Asp-N cleavage specificity are highlighted in green.

FIGURE 6. Identification of the CII169 degradation product. A, Western blot analysis of insertion mutants show that the degradation product consists of the N-terminal 132–142 amino acids of CII169. A 5-amino acid insertion immediately following residue 4 (4ins) or residue 131 (131ins) is evident in both the full-length protein (filled arrowheads) and the degradation product (open arrowheads). A 5-amino acid insertion immediately following residue 166 (166ins) is evident in the full-length protein, but not the degradation product. The degradation product migrates at a lower apparent molecular weight than a CII142 truncation. Separated images are from independent gels. Solid vertical lines indicate separation of non-contiguous lanes on the same gel and blot. B, in gel Asp-N digest and LC-MS/MS analysis of the CII169 degradation product. Sequence coverage is indicated by bold type. Lines under the sequence indicate peptides detected by LC-MS/MS. Peptides with C-terminal sequences that do not conform to the Asp-N cleavage specificity are highlighted in green.
Charaterizing CII135—Having determined that the in vivo degradation product of CII169 corresponds to a CII135 truncation, we sought to characterize this truncation. Given the relative abundance of the degradation product in vivo, the activity and function of the CII135 form has important implications for the physiological role of 186 CII, but equally could provide further insights into the structure and function of the full-length CII169 protein. Reporter assays using pCIIR show that CII135 exhibits significantly reduced DNA binding, despite expressing at higher cellular concentrations relative to both CII169 and CII145 (Fig. 7A). Correspondingly, in vivo pE reporter assays show that CII135 exhibits some 97% less activity at maximum induction than CII169 (Fig. 7B). Nonetheless, some residual activity is still clearly detectable at higher levels of protein expression (Fig. 7B, inset), consistent with other truncations within the putative self-association domain isolated by linker insertion mutagenesis.

Thus, we expect that the primary mechanism by which truncations within the C-terminal domain lose activity is by loss of self-association and cooperativity in DNA binding, or by direct loss of an independent DNA-binding function. Addressing this question, we mutated residues Val-36 and Gln-37 of CII169. These amino acids are at positions 12 and 13 of the helix-turn-helix according to the positioning scheme adopted by the motif prediction algorithm (11), which contributes strongly to sequence specificity (43, 44), but tolerate a diversity of amino acids. In an effort to minimize the chances of causing structural defects, we mutated Val-36 and Gln-37 to other amino acids that are commonly found at positions 12 and 13 of helix-turn-helix proteins, resulting in the CII169V36E/Q37S mutant. This mutant does not exhibit any detectable activity at pE (Fig. 7B), and does not repress pCIIR, despite being observable in cellular extracts by Western blot (Fig. 7A). These results suggest that there is no significant DNA-binding function encoded by regions of the protein outside of the helix-turn-helix motif.

Examining the loss of self-association hypothesis directly, we used size-exclusion chromatography coupled with multiangle light scattering analysis (SEC-MALS) to determine the native, solution state molecular weights of purified His-tagged CII135, CII145, and CII169 (Fig. 7C). The observed peak molecular masses of CII169 (86.0 ± 3.4 kDa; 95% CI) and CII145 (82.9 ± 9.9 kDa; 95% CI) are consistent with a tetrameric native form. In contrast, the observed peak molecular mass of CII135 was 35.5 ± 2.8 kDa (95% CI), consistent with the theoretical dimer mass of 34.9 kDa. Thus we conclude that the CII135 truncation impairs CII self-association, presumably by disruption of the C-terminal domain.

DISCUSSION

Prokaryotic transcriptional activators employ a variety of mechanisms to increase transcription from their target promoters. Understanding a variety of activators is important to detecting patterns in how or why different activation mechanisms are employed, in terms of the constraints and features of their action. That is, it helps us to gain insights into what, if any, evolutionary pressures have steered organisms toward particular solutions to the problem of transcriptional control. The relationship between the structure and function of these activators is a key component of understanding the underlying mechanisms.

In this study, we find evidence that the DNA-binding activity of 186 CII is conferred by the N-terminal region, whereas the C-terminal region is responsible for CII self-association. We have not obtained direct evidence that both regions fold independently, so denote these as functional rather than structural domains. Attempts to express the His-tagged N-terminal domain (residues 1–81) and C-terminal domain (residues 82–145) independently have been unsuccessful. It is unclear whether this is due to the choice of domain boundary, an effect of the His8 affinity tag, or some level of structural dependence between the domains. Our data concerning CII135 suggest that the N-terminal domain at least is substantially structurally independent, but the independence of the C-terminal domain is less clear.

Our earlier work investigating CII self-association by analytical centrifugation has indicated a CII to DNA stoichiometric ratio of 2.5 under conditions of CII excess and saturated DNA binding sites (6). That study concluded that CII binds DNA as a
redundancy. If, for instance, CII Glu-46 can contact two alternative residues alone may not produce a defect, whereas mutation of Glu-46 would produce a strong defect, as observed. Under this model, the behavior of R115L CII145 suggests that this epitope does indeed interact with $\sigma^{70}$. However, the Lys-593 $\sigma^{70}$ variants do not reduce pE activation by wild-type or R115E CII145, the inhibitory role of $\sigma^{70}$ Lys-593 must be reliant on prior rearrangement of the interface by the CII Glu-46 or Arg-17 mutations. According to this model, the key residue(s) on the $\sigma^{70}$ side of the wild-type interface remain unidentified, because our analysis was restricted to a specific region of $\sigma^{70}$. We favor the first model for its relative simplicity, but cannot fundamentally distinguish between them given our observations. Importantly, however, both models rely on close intramolecular contact between CII and $\sigma^{70}$, giving a strong indication that the Glu-46 activation epitope of CII acts via a $\sigma^{70}$-dependent mechanism.

De novo and homology structure prediction approaches yield models of the N-terminal domain with moderate levels of confidence, but significantly, these independent approaches produce very similar overall folds, with a 3.90-Å backbone root mean square deviation, excluding terminal regions without secondary structure (residues 8–74). The Rosetta de novo model predicts a solvent-exposed helix-turn-helix, positioned ready for DNA binding (Fig. 8A). Importantly, it predicts that Arg-17 and Glu-46 lie adjacent to each other in the three-dimensional fold, consistent with our experimental data showing that mutations at these residues form part of the same activation epitope. With the helix-turn-helix docked into the major groove, this lies on one side of the protein, positioned in a way that makes contact with $\sigma^{70}$ plausible (46) (Fig. 8B).

FIGURE 8. Promoter activation by 186 CII. A, a Rosetta de novo model of the CII N-terminal domain predicts a solvent-exposed specificity helix of the helix-turn-helix motif (orange), whereas mutations identified in our activation mutant screen (depicted in green) cluster on one side of the domain. B, when the specificity helix of CII is docked into the major groove of DNA, residues Arg-17 and Glu-46 (green) are positioned ready for contact with $\sigma^{70}$ region 4 (yellow), which binds at the −35 region of the promoter. The structure of DNA-bound $\sigma^{70}$ region 4 is from PDB code 1RIO (46). C, schematic representation of our model of promoter activation by CII. Under this model, the CII NTD contacts $\sigma^{70}$, whereas contacts between the CII CTD and one or both CTDs stabilize them on the DNA in between the DNA half-sites at −38 and −58. Only the CII DNA-binding dimer is shown.
Promoter Activation by 186 CII

With these models of self-association and activation in mind, it is worth re-examining prior evidence of the structural arrangement of CII and RNAP on the pE promoter. DNase I footprinting experiments using CII1169 alone, or CII1169 together with RNAP are instructive (4, 6). Both half-sites and the region between them are protected in the presence of CII1169 alone, with the exception of a cleavage enhancement midway between the half-sites. In the presence of RNAP, two changes are evident. First, there is protection of the region downstream of the CII half-sites, extending to around +20 relative to the transcription start site. This is consistent with recruitment of \( \sigma^{52} \) and the RNAP core enzyme to the promoter by the N-terminal helix-turn-helix domain bound at the promoter proximal CII half-site. The second change is a shift in the enhancement between the CII half-sites. The current study suggests an enhancement between the CII half-sites. The current study directly via a resulting CTD must lie physically between the NTDs. Combining these observations, we arrive at a model of the promoter architecture in which CII stabilizes one or both αCTDs on the DNA region between the CII half-sites (Fig. 8C). To our knowledge, this arrangement of factors and interactions at a promoter has not been previously observed.

Our finding that CII is rapidly degraded in vivo extends our knowledge of the functional similarity between 186 CII and λ CII. In both cases, degradation of the protein is promoted by a C-terminal tail (47) and results in similarly short half-lives of around 2 min (8, 35). Given the role of the host protease FtsH in degradation of λ CII, and the importance of λ CII proteolysis to the frequency of lysogeny of λ phage (9), it would be interesting to investigate whether these are also conserved properties in 186 CII. FtsH-mediated proteolysis does not typically yield observable reaction intermediates (48), in contrast to our observation of a specific degradation product for 186 CII. Thus we speculate that 186 CII may be degraded by an alternative protease, offering the potential to explore whether the choice of protease used for degrading CII is important, as opposed to the simple fact of degradation itself. The mechanisms by which λ CII and 186 CII activate transcription also extend the functional, but not structural homology of their host phage, because both are thought to stabilize the αCTD and \( \sigma^{52} \) but in quite different ways. λ CII binds as a tetramer to direct repeat half-sites separated by only a single turn of the DNA helix, overlapping the \( \sigma^{52} \) binding site (49). λ CII is thought not to contact \( \sigma^{52} \) directly, but rather contacts αCTD, stabilizing it in a position conducive to \( \sigma^{52} - \alphaCTD \) contacts (50, 51). According to this model, λ CII recruits RNAP directly via the αCTD and indirectly via a resulting αCTD–\( \sigma^{52} \) interaction. This functional conservation likely stems from a physiological requirement for the functionally analogous CII proteins to be potent activators.

It remains unclear whether the particular arrangements of factors at the promoter confer specific advantages or properties on the promoters and what those advantages might be. One possibility is that αCTD binding between the CII half-sites on pE might result in greater activation due to the αCTD contact, by allowing αCTD to contact the promoter in close proximity to \( \sigma^{52} \). In the case of αCTD activation via an UP element contact, the strength of activation is inversely related to the distance of the UP element from \( \sigma^{52} \) (52), presumably due to the lower entropic cost of a minimally extended linker. Our model of pE activation by CII suggests that it is plausible that both αCTDs directly contact CII and DNA. In contrast, whereas both αCTDs are known to be involved with cAMP receptor protein-mediated activation, structures of the cAMP receptor protein–αCTD-DNA complex show that only one αCTD makes direct contact with cAMP receptor protein (53). Thus the unusual pE promoter architecture may serve to achieve greater αCTD-mediated activation through independent stabilization of both αCTDs on the DNA (Fig. 8C), without the need for sequential binding events and without compromising contacts with \( \sigma^{52} \).

Another possible reason for the adoption of this promoter architecture derives from the biological importance of rapid and effective proteolytic degradation of CII in the bacteriophage. Helix-turn-helix proteins bound as dimers in adjacent major grooves are in close physical proximity, which can lead to some cooperativity in DNA binding that is independent of a separate self-association domain, as in the case of λ CI (54). We have shown that proteolysis of CII is dependent on its C-terminal tail, and yields a specific, inactive product comprised of the first 135 residues. Based on the results presented here, the degradation product has lost one self-association interface, and this almost completely destroys its DNA-binding activity. By separating in space the half-sites, and thus DNA binding domains of CII, there is no possibility of residual interaction and cooperativity in the intact NTDs. We could expect that this would lead to a more sensitive response of pE activity to proteolytic cleavage of CII. Encoding an activation epitope within the CII CTD, which becomes disrupted by degradation, would further enhance this sensitivity, which may be the key functional feature of this promoter architecture.

Acknowledgments—We thank Steven Busby, Richard Ebright, Seth Darst, Rachel Schubert, and eResearchSA for experimental tools; Daan van der Neut for preliminary data; and Julian Pietsch, Rachel Schubert, and Barry Egan for discussions.

REFERENCES
1. Ptashne, M. (2004) A Genetic Switch: Phage Lambda Revisited, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Trusina, A., Sneppen, K., Dodd, I. B., Shearwin, K. E., and Egan, J. B. (2005) Functional alignment of regulatory networks: a study of temperate phages. PLoS Comput. Biol. 1, 674
3. Lamont, I., and Richardson, H. (1993) Genes for the establishment and maintenance of lysogeny by the temperate coliphage 186. J. Bacteriol. 175, 5286–5288
4. Neufang, P. J., Shearwin, K. E., Camerotto, J., and Egan, J. B. (1996) The CII protein of bacteriophage 186 establishes lysogeny by activating a promoter upstream of the lysogenic promoter. Mol. Microbiol. 21, 751–761
5. Neufang, P. J., Shearwin, K. E., and Egan, J. B. (2001) Establishing lysogenic transcription in the temperate coliphage 186. J. Bacteriol. 183, 2376–2379
6. Shearwin, K. E., and Egan, J. B. (2000) Establishment of lysogeny in bacteriophage 186: DNA binding and transcriptional activation by the CII protein. J. Biol. Chem. 275, 29113–29122
7. Hoyt, M. A., Knight, D. M., Das, A., Miller, H. L., and Echols, H. (1982) Control of phase λ development by stability and synthesis of CII protein: role of the viral CII and host hflA, himA and himD genes. Cell 31, 565–573
8. Rattray, A., Altuvia, S., Mahajna, G., Oppenheim, A. B., and Gottesman,
Promoter Activation by 186 CII

(2004) Role of the RNA polymerase alpha subunits in CII-dependent activation of the bacteriophage λ pE promoter: identification of important residues and positioning of the α C-terminal domains. Nucleic Acids Res. 32, 834–841

51. Jain, D., Kim, Y., Maxwell, K. L., Beasley, S., Zhang, R., Gussin, G. N., Edwards, A. M., and Darst, S. A. (2005) Crystal structure of bacteriophage λ CII and its DNA complex. Mol. Cell 19, 259–269

52. Meng, W., Belyaeva, T., Savery, N. J., Busby, S. J., Ross, W. E., Gaal, T., Gourse, R. L., and Thomas, M. S. (2001) UP element-dependent transcription at the Escherichia coli rrnB P1 promoter: positional requirements and role of the RNA polymerase α subunit linker. Nucleic Acids Res. 29, 4166–4178

53. Benoff, B., Yang, H., Lawson, C. L., Parkinson, G., Liu, J., Blatter, E., Ebright, Y. W., Berman, H. M., and Ebright, R. H. (2002) Structural basis of transcription activation: the CAP-α CTD-DNA complex. Science 297, 1562–1566

54. Pabo, C. O., and Lewis, M. (1982) The operator-binding domain of λ repressor: structure and DNA recognition. Nature 298, 443–447