Robustness of a gene regulatory circuit

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Complex interacting systems exhibit system behavior that is often not predictable from the properties of the component parts. We have tested a particular system property, that of robustness. The behavior of a system is termed robust if that behavior is qualitatively normal in the face of substantial changes to the system components. Here we test whether the behavior of the phage λ gene regulatory circuitry is robust. This circuitry can exist in two alternative patterns of gene expression, and can switch from one regulatory state to the other. These states are stabilized by the action at the O_R region of two regulatory proteins, CI and Cro, which bind with differential affinities to the O_{R1} and O_{R3} sites, such that each represses the synthesis of the other one. In this work, this pattern of binding was altered by making three mutant phages in which O_{R1} and O_{R3} were identical. These variants had the same qualitative *in vivo* patterns of gene expression as wild type. We conclude that the behavior of the λ circuitry is highly robust. Based on these and other results, we propose a two-step pathway, in which robustness plays a key role, for evolution of complex regulatory circuitry.

Keywords: evolution of gene regulation/genetic switch/
lambda repressor/lambda phage/system behavior

Introduction

It is a truism in describing complex systems that the whole is more than the sum of its parts. But what does this mean, and how can we characterize and study this higher order of complexity? Complex systems have 'emergent' behavior that arises from interactions among their components. A familiar example is feedback, in which the output of the system, or more commonly of a part of the system, provides input at a later time, and this new input in turn modifies the behavior. Other system properties deal with the behavior of the system as a whole, and are particularly relevant in cases such as gene regulatory circuits, in which a system can adopt more than one alternative stable state. Examples of such properties are stability (the ability of a system to maintain its state in the face of small perturbations) and threshold behavior (the property of maintaining a constant state below a particular input value, and a change to a different state when the input exceeds that threshold).

Our intuition is not a reliable guide for predicting these and other system properties, due to factors such as feedback, non-linearity and the extensive interlocking of system components. Nonetheless, a full understanding of a biological system such as a gene regulatory circuit demands a description of its system behavior. This description should include predictions about the temporal course of events in dynamic systems, about the effects of perturbations on the system, and about the effects of changes in the system components. In principle, computer simulations can provide insights into these areas, but this approach is useful only in the few cases where knowledge of the system is advanced enough that we know most or all of the components and their interactions (McAdams and Shapiro, 1995; Barkai and Leibler, 1997). Even in these cases, stochastic behavior due to small numbers of molecules can greatly complicate the analysis (McAdams and Arkin, 1997). A related approach (Savageau, 1974; Hlavacek and Savageau, 1995) is mathematical analysis of idealized systems, followed by predictions of their behavior and of the effects of modifications. A complementary approach, which is taken here, is to explore system behavior experimentally.

The present work focuses primarily on another system property, that of robustness. This term has various informal meanings, but in control theory it is defined as the ability of a system to continue functioning in the face of substantial changes to its components (Savageau, 1971). This system property, often termed 'parameter sensitivity', was introduced into molecular biology (Savageau, 1971, 1974) and developed further by Savageau and colleagues (Shiraishi and Savageau, 1992; Hlavacek and Savageau, 1995) (see also Discussion). In biology, it is plausible, although untested, that robustness is an important factor in tolerating genetic polymorphism and in the evolution of complex regulatory systems (Barkai and Leibler, 1997; Hartwell, 1997). Recent experimental work (Alon *et al.*, 1999) has shown that the chemotaxis system of *Escherichia coli* shows robust behavior.

Robustness is qualitatively different from stability. The latter term refers to the ability of a system behavior to withstand chance fluctuations in the levels of components, such as regulatory proteins, in the absence of genetic changes to these components. Such fluctuations might be caused by environmental perturbations, or by stochastic effects due to variable efficiency of transcription or translation, effects that are particularly likely when cells contain small numbers of molecules (McAdams and Arkin, 1997). Robustness, in contrast, refers to the effects of genetic changes in the components.

In this work, we have analyzed the robustness of a well-characterized gene regulatory circuit, that of phage λ . Gene regulatory circuits often persist in alternative stable states, as in the λ case, and it is of interest to know

whether the ability of these circuits to exhibit these stable states is delicately balanced, i.e. whether the behavior requires exactly the details of the system that we find in nature, or alternatively whether these properties are robust, in the sense that the details can change significantly without completely disrupting the system behavior (Barkai and Leibler, 1997; Alon *et al.*, 1999). λ is an attractive system for such a test, because the regulatory circuitry has been studied in great detail. The patterns of gene expression are well understood; moreover, the regulatory proteins, their interactions with their binding sites and the consequences of binding are well characterized, both genetically and structurally.

A critical test of robustness would be to alter parameters of the system that appear likely to play central roles in determining regulatory states. Important quantitative parameters in gene regulatory circuits include such elements as promoter strength, stability of proteins, strength of cooperativity of DNA binding and affinities of DNA-binding proteins for their sites. Because the affinities of two DNA-binding proteins for their sites are thought to be crucial for proper operation of the λ circuitry (Ptashne, 1992), we chose to perturb them as a test of robustness.

The central events that control and stabilize the regulatory states occur at a complex site termed the O_R region (Figure 1A). This region contains two promoters: the lytic promoter P_R , from which RNA polymerase transcribes *cro* and several early lytic genes, and P_{RM} , which transcribes *cI* in a lysogen. O_R also contains three sites to which both Cro and CI bind. However, they do not bind equally well to all three sites, and their patterns of binding are believed to help stabilize the regulatory states (Ptashne, 1992). Each stable state involves the continued expression and action of one protein, and the repression and absence of the other. CI binds tightly to O_R1 and weakly to O_R3 ; CI also binds cooperatively to O_R1 and O_R2 (as in the $2^{\wedge}1$ pattern, Figure 1D; see also Figure 2). In the lysogenic state, CI represses expression of *cro* and lytic genes from P_R , and the repressor molecule bound to O_R2 stimulates *cI* expression from P_{RM} . Conversely, Cro binds weakly to O_R1 and O_R2 and tightly to O_R3 (Figure 2); hence, in the lytic state, Cro represses P_{RM} fully, but represses P_R only at high concentrations, such as those found late in infection, so that early gene expression is reduced at later times. Occupancy of O_R3 by Cro is also important in the process of prophage induction. This change of state from lysogenic to lytic, often called the 'genetic switch', occurs when the host SOS system is induced, leading to cleavage and inactivation of CI, in turn resulting in synthesis of Cro; repression of P_{RM} by Cro then makes the switch irreversible.

Work with two other phages similar to λ has shown that the *in vitro* binding patterns of CI and Cro homologues at the cognate O_R regions differ from the well-studied λ case. In phage $\phi 80$, CI binds in the order $O_R2 > O_R3 > O_R1$, although cooperativity has not been analyzed in detail; Cro binds in the order $O_R1 \approx O_R3 \gg O_R2$ (Ogawa *et al.*, 1988). In phage HK022, the sequences of the core of operators O_R1 and O_R3 are the same (Oberto *et al.*, 1989). HK022 CI binds somewhat more tightly to O_R1 than to O_R3 , but the primary mechanism for ensuring occupancy of O_R1 and O_R2 is that the spacing between these sites supports a much higher degree of cooperativity than that

Fig. 1. Maps and sequences of O_R . (A) Map of the O_R region. The P_{RM} and P_R promoters transcribe *cI* and *cro*, respectively; locations of their -35 and -10 regions are shown. Map is to scale, except that the AUG codon of *cro* lies 26 bp from O_R1 . The sites mutated in O_R1 and/or O_R3 are indicated by vertical lines and do not change the -35 or -10 regions of either promoter. (B) Sequences of O_R3 , O_R1 and the hybrid O_R3' site. The three differences are in bold type; O_R3' differs from O_R3 in the underlined position, which has the C residue found in O_R1 . The sequence of O_R3 shown is reversed from that in the map above. (C) Schematic depiction of variant O_R regions. Positions that were changed to the base found in the other operator are indicated by vertical lines. WT, wild type. (D) Postulated pattern of occupancy by CI in lysogens of symmetrical variants. The N-terminal domain of CI binds DNA, and its C-terminal domain mediates dimerization and cooperativity. Cooperative binding of CI to O_R is 'alternate pairwise'; the dimer at O_R2 can contact one at O_R3 or at O_R1 but not both simultaneously (Ptashne, 1992). Complexes denoted $3^{\wedge}2$ and $2^{\wedge}1$ have the indicated binding patterns; $2^{\wedge}1$ forms on wild-type O_R . The cooperativity parameters for $2^{\wedge}1$ and $3^{\wedge}2$ are about the same (Koblan and Ackers, 1992). When the sites at O_R1 and O_R3 are the same, the two complexes should be about equally stable, and hence be present about the same fraction of the time. This expectation is consistent with footprinting data (Figure 2) for O_R323 and $O_R3'23'$, which showed almost complete occupancy of O_R2 and about 50% occupancy of flanking sites at intermediate and high CI levels. In contrast, with O_R121 we expect substantial occupancy of the flanking site (such as the O_R1 site in $3^{\wedge}2$ in the absence of cooperativity, since this site is a strong binding site. Footprinting of O_R121 (Figure 2) showed almost complete occupancy of the flanking sites at CI levels a few-fold higher than needed to give full occupancy of O_R2 . (E) Map of pDWW1. The detailed portion of the map, corresponding to the insert, is to scale; the pBS(-) vector is not. Sites marked *EcoRI/X*, *X/BglIII*, and *NsiI/PstI* denote joints that destroyed restriction sites; X denotes an end made with hybridized synthetic oligos. The O_R sites are shown as black boxes. Truncated ends of the *cI* and *cro* genes are indicated by " " the location of the synthetic *trp* attenuator is shown. Inserts were sequenced using a T7 or T3 primers hybridizing to the left or right, respectively, of the insert.

Fig. 2. Binding of Cro and CI to variant O_R regions. DNase I footprinting was carried out as in Methods. Upper left, wild type (WT); upper right, O_{R121} ; lower left, O_{R323} ; lower right, $O_{R3'23'}$. Wedges denote increasing concentrations of protein; for CI, concentrations (expressed as total monomer added) were 2.5, 5, 10, 20, 40, 80, 160 and 500 nM; for Cro, 5, 10, 20, 40, 80, 160, and 500 nM. Samples in lanes marked with ‘–’ above and below contained no added Cro or CI. The positions of O_{R1} , O_{R2} and O_{R3} are indicated and were inferred from a G+A marker lane (Muro *et al.*, 1993) (not shown).

between O_{R2} and O_{R3} (Carlson and Little, 1993). HK022 Cro binds about equally well to O_{R1} and O_{R3} (Carlson, 1992). One might conclude from these examples that regulation at O_R is robust in lambdoid phages, and that a test in λ is superfluous. However, regulation in these two other phages has not been analyzed in the depth that has been done for λ . The details of the regulation in $\phi 80$ and HK022 may differ from those in λ , e.g. there may be differences in expression levels, in mRNA or protein stability, or in transcription patterns, making a direct comparison with λ difficult.

As a test of robustness in λ , we disrupted the binding pattern of Cro and CI by allowing equal binding to O_{R1} and O_{R3} . One or both sites were changed by site-directed mutagenesis, and phage carrying the altered O_R regions were isolated and characterized. Our results demonstrate that the qualitative pattern of λ gene regulation persists in the face of these changes, and we conclude that the behavior of the λ gene regulatory circuit is highly robust.

Results

Approach

To afford equal binding of Cro and CI to O_{R1} and O_{R3} , we made three ‘symmetrical’ variants in which both sites

had the same sequence (Figure 1B and C), using site-directed mutagenesis on plasmids. O_{R1} and O_{R3} differ in three positions, and these positions do not overlap the –35 or –10 regions of the two promoters in this region, P_R and P_{RM} . Hence, changes in these positions should affect binding of Cro and CI, but not of RNA polymerase (although we have not tested effects on promoter strength directly). The O_{R1} sequence was changed to that of O_{R3} , creating an O_R region we term O_{R323} ; O_{R3} was changed to O_{R1} , yielding O_{R121} ; and both sites were changed to a hybrid site we term $O_{R3'}$, yielding $O_{R3'23'}$.

DNase I footprinting verified that Cro and CI bound to wild-type O_R in the pattern described above (Figure 2A). With the variants, Cro bound about equally tightly to the two flanking sites on each variant O_R site, in contrast to the wild type (Figure 2, left side of each panel). CI showed a more complicated pattern of binding to the variant sites (Figure 2, right side of each panel), most readily seen on the O_{R323} and $O_{R3'23'}$ templates: the central site was almost fully occupied and each flanking site was partially protected (see also Figure 1D legend). This pattern is consistent with the model (Figure 1D) that a mixture of two alternative pairwise combinations is present (see also Discussion).

Wild-type and variant O_R regions were then crossed

onto λ by *in vivo* recombination between these plasmids and λ *lv3*, which forms clear plaques; wild-type and variant phages, termed λO_R^+ , λO_R323 , λO_R121 and $\lambda O_R3'23'$, were isolated by their turbid-plaque phenotype (see Materials and methods). The biological properties of the variants were then compared with those of the wild type.

Behavior of symmetrical variants

By the following qualitative criteria (quantified below), each of the three variants had functional regulatory circuits. First, the variants could grow lytically. Secondly, they readily formed lysogens that were highly stable, indicating that they could establish and maintain the lysogenic state. Finally, these lysogens readily underwent prophage induction (Roberts and Devoret, 1983; Little, 1996) upon irradiation with ultraviolet (UV) light, indicating that they could switch from the lysogenic to the lytic state upon induction of the SOS system. We conclude that the differential affinities of Cro and CI for the O_R operators are not necessary either for stable lysogeny or for the genetic switch. More importantly, the output of the λ regulatory circuitry is robust, in that substantial changes in the circuitry allow it still to function.

The following data document the above summary, and in addition provide evidence that wild-type grows better than the O_R variants under laboratory conditions. First, to measure lytic growth, we measured burst sizes (phage per infected cell) and the timing of phage production after single infection (Table I). $\lambda O_R3'23'$ had about the same burst size as wild type; that of λO_R121 was ~30% lower, and λO_R323 had both a lower burst size and a delay of ~10 min in producing phage. This phage also formed small plaques. Secondly, all the variants lysogenized readily (Table I); λO_R323 did so at a reduced frequency. Thirdly, we assessed stability of lysogens by measurement of free phage in cultures of lysogens. Breakdown of the lysogenic state should lead to lytic growth and production of free phage. In *recA*⁺ hosts, RecA-dependent spontaneous induction occurs in a small fraction of cells in the absence of overt DNA damage (Roberts and Devoret, 1983), leading to a relatively high level of free phage (Table I). This level was higher with the variants. To assess the intrinsic stability of lysogens in the absence of complications from the SOS system, free phage were measured with *recA*⁻ lysogens. The wild type gave a very low level. We estimate (Table I legend) that cultures of wild-type lysogens contained roughly one cell in 2.5×10^6 that had switched to the lytic state. For the variants, $\lambda O_R3'23'$ had about the same level of free phage as wild type, while λO_R121 and λO_R323 had higher levels. Even for the least stable variant, λO_R323 , the lysogenic state broke down in only about one cell in 5×10^4 , still an impressive degree of stability.

Finally, to measure prophage induction, lysogens were irradiated with varying doses of UV light and phage production was measured (Figure 3). All lysogens gave efficient induction. All but $\lambda O_R3'23'$ gave burst sizes approximately equal to that of wild type. The UV doses required to give induction differed, with the wild type requiring the highest level of UV (Figure 3). The variants could be said to have a hair-trigger. As known previously (Bailone *et al.*, 1979), the wild type showed a threshold

Fig. 3. UV dose responses for prophage induction. Lysogens of each phage were irradiated at the indicated dose of UV light, and the number of phage per input cell was measured (see Materials and methods).

response, giving little induction at low UV doses. Importantly, λO_R121 and $\lambda O_R3'23'$ also showed a threshold response (as did λO_R323 at lower doses; data not shown), indicating that the differential affinities are not required for a threshold to exist, and that the threshold behavior of the system is also robust with respect to changes at O_R .

To summarize, each of the variants grew less well than wild-type under one of these conditions. λO_R323 gave relatively poor lytic growth, and λO_R121 was also somewhat defective. $\lambda O_R3'23'$ gave a reduced yield after prophage induction. These growth defects would plausibly make the variants less fit.

Evolution of a symmetrical variant toward wild type

As noted above, λO_R323 formed small plaques. This phenotype allowed strong selective pressure for better lytic growth. Stocks of λO_R323 frequently contained secondary variants that made large plaques. We sequenced the O_R region of 21 large-plaque variants. All of these had changes in the O_R3 site located at the position of O_R1 (Figure 4). Most variants formed clear plaques, presumably because binding of both Cro and CI to the site was impaired (see Discussion). Several variants formed turbid plaques; among these, three of five tested had a change to O_R3' , giving $\lambda O_R323'$. $\lambda O_R323'$ also formed stable lysogens.

These findings have three implications: first, at the mechanistic level, the change from O_R3 to O_R3' is known (Hochschild *et al.*, 1986) to weaken the binding of Cro (see also Figure 2), implying that the growth defect of λO_R323 results from an inappropriately early and strong repression of P_R due to strong binding of Cro to this site; this restricts early lytic gene expression more than in the wild type, limiting phage production. The change from O_R3 to O_R3' also strengthens somewhat CI binding to the site, but CI need not be expressed during lytic growth, so tighter CI binding should not be responsible for the improved growth. Secondly, λO_R323 has evolved toward wild type under selective pressure. O_R3 and O_R1 differ by three positions; $\lambda O_R323'$ has taken one of the three steps from λO_R323 to wild type. Finally, the other two turbid-plaque variants move O_R further away from wild-type λ ; they contain four changes in the O_R1 site relative

Table I. Properties of O_R variants

Phage	Lytic growth		Lysogenization frequency	Relative CI level in lysogen	Free phage per lysogenic cell		Estimated fraction of <i>recA</i> [−] lysogens that have switched to lytic state	Burst size after UV induction
	burst size	latent period			<i>recA</i> ⁺	<i>recA</i> [−]		
λ ⁺	56	36 min	63%	≅100%	1.0×10 ^{−3}	1.2×10 ^{−5}	4×10 ^{−7}	76
λO _R 121	38	36 min	57%	25–30%	5 ×10 ^{−3}	8×10 ^{−5}	3×10 ^{−6}	70
λO _R 323	26	46 min	33%	60–75%	1.0×10 ^{−2}	5.6×10 ^{−4}	2×10 ^{−5}	68
λO _R 3'23'	57	36 min	60%	50–60%	1.7×10 ^{−3}	0.7×10 ^{−5}	5×10 ^{−7}	33

Tests are given in Materials and methods and discussed in the text. CI levels were particularly low in λO_R121 lysogens; in these lysogens, we would expect that on the 2[^]1 complex (Figure 1D) the site at O_R3 would bind CI relatively well (see Figure 2), so that P_{RM} would be repressed at low CI levels. The burst size after UV induction was taken as the highest value in Figure 3. To estimate the fraction of *recA*[−] cells undergoing spontaneous induction, we assumed that burst sizes would be the same as after UV induction of *recA*⁺ strains (last column), and that lysis would occur 60 min after the cell switched its state. A computer simulation was carried out in which a given fraction of cells switched to the lytic state per minute, and these cells released the given burst 60 min later; free phage were tabulated and divided by cell numbers, given the observed doubling time of 34 min. Comparison with the measured values of free phage gave a fraction switching state per min; the numbers of cells switching state each min for 60 min were summed and compared with the total number of cells present at 60 min.

Fig. 4. Mutational changes in the O_R3 site at the position of O_R1 in λO_R323. Large-plaque variants were isolated, and the O_R regions were sequenced (Materials and methods). The last variant shown made medium-sized plaques. The plaque morphology of each isolate is indicated. Listed at the right are the ΔΔG values determined for these changes in the context of O_R1; values for Cro (Takeda *et al.*, 1989) and CI (Sarai and Takeda, 1989) were taken from Figure 1 of the cited references. It is likely, however, that in the context of O_R3 (with three changes in the left half-site) these values would differ, particularly for changes in the left half of the operator. Positions in the operator are numbered as indicated above the sequence; the −10 region of P_R is shown.

to wild type (Figure 4). We suggest that their existence raises the possibility that the λ regulatory circuit could evolve into something with qualitatively similar behavior but different mechanisms.

Discussion

Robustness

Robust behavior of a system is defined as the ability of that behavior to tolerate changes in the system parameters.

Though other usages are possible, for the sake of discussion we shall apply the term ‘robustness’ to the behaviors or outputs of a system, not to the system itself. Moreover, we shall term these outputs robust with respect to changes in particular parameters.

Robustness has been defined quantitatively in mathematical terms (e.g. Savageau, 1971; history reviewed in Shiraishi and Savageau, 1992; Hlavacek and Savageau, 1995), in a treatment developed mostly for systems with behavior that changes smoothly with the changes in parameters. In principle, this treatment could be applied to quantitative changes in outputs such as burst sizes and threshold set-points. However, it is difficult to do so directly in the present case, because in all our mutants two different parameters (affinities of Cro and CI for the mutated operator) were changed simultaneously, so that the individual sensitivities of these two parameters cannot be assessed unambiguously (M.Savageau, personal communication; see Materials and methods). Moreover, this approach does not pertain to the qualitative properties of systems with switch-like behavior, and it is uncertain how to apply them to such cases.

Nonetheless, switches and other systems with alternative states are prevalent throughout biology, and the concept of robustness is directly applicable to them as well. For such systems, we take the view that it is the qualitative patterns of system behavior that are crucial, rather than relatively small quantitative effects on system properties. If the behaviors are qualitatively the same following substantial changes in parameter values, we shall term them robust.

In the case of λ, the important system properties are qualitative and deal with alternative gene regulatory states: the system allows two different patterns of gene expression; the lysogenic pattern is indefinitely stable, and the system can undergo the genetic switch upon induction of the SOS system. In addition, the genetic switch shows threshold behavior in its response to graded amounts of DNA damage.

Our data provide two lines of evidence that these properties of the λ gene regulatory circuitry are robust with respect to changes at O_R: (i) the differential affinities for binding of Cro and CI to the O_R operators are not required to allow qualitatively normal operation of the regulatory circuitry; and (ii) threshold behavior is observed

in the symmetric O_R variants. These findings have two broad and general implications. First, the work provides strong support to previous predictions (Savageau, 1974; Barkai and Leibler, 1997) and a recent experimental demonstration (Alon *et al.*, 1999) that biochemical networks are not delicately balanced, but can continue to function over a range of parameters. Secondly, our work supports the view that intuition is often not the best guide to predicting the behavior of complex systems. Despite the great intuitive appeal of the model for the crucial role of differential repressor binding, this feature is evidently not an essential aspect of λ regulation; instead, as we discuss below, it is likely a fine-tuning of the circuitry for optimal behavior.

Several other examples of robustness in biology have been provided by various complementary approaches. Mathematical analyses by Savageau and colleagues have predicted, for example, that gene regulatory systems would be robust (Savageau, 1974), and have explored existing models of metabolic circuits for weak points that are not robust (Shiraishi and Savageau, 1992). In *E.coli*, one aspect of the chemotaxis system, the ability to adapt to stimulation by a high level of attractants, is highly robust with respect to changes in concentrations of the molecules involved, whereas quantitative changes in the time taken for adaptation were less so (Alon *et al.*, 1999). In most of these experimental tests, the perturbation was elevated levels of one protein component of the system. In the MAP kinase cascade, computer analysis predicts that the switch-like behavior of the cascade is robust with respect to the concentrations and K_m s of the phosphatases and kinases (Huang and Ferrell, 1996).

Finally, we note that there are limits to the robustness of the λ circuitry. We made a phage (λO_R123) in which O_R1 and O_R3 are switched (unpublished data). This phage could not lysogenize, and formed such tiny plaques that stocks were invariably overgrown with secondary variants. For this reason, it has not been characterized further.

Mechanisms

How can we explain at the mechanistic level the ability of the λ regulatory circuitry to work when O_R3 and O_R1 are identical in sequence? The proposed explanations differ for each aspect of the circuitry. We shall assume for the sake of discussion that the mutations affect only the binding of Cro and CI to O_R , and not the properties of the P_{RM} and P_R promoters, but we have not tested this assumption directly by analysis of promoter strengths.

First, the lysogenic state must be highly stable. It persists for many generations, both in wild-type and the O_R variants. The footprinting patterns (Figure 2), and known properties of CI, lead us to suggest a mixed occupancy pattern of binding. We postulate that the pattern of *in vivo* occupancy of O_R is composed of roughly equal parts of two complexes, 2^1 and 3^2 (Figure 1D), each involving pairwise cooperative binding, and that these complexes are dynamic enough that in a given cell each complex is present roughly half the time. The sum of this mixed occupancy pattern would be a regulatory state like wild type, with P_{RM} partly on and P_R off (Figure 1D). This model predicts lower levels of CI in lysogens of the variants, as observed (Table I).

Secondly, the ability of a lysogen to undergo the genetic

switch depends primarily on reductions of CI levels due to RecA-mediated cleavage; when CI is reduced below a certain level, P_R becomes derepressed and the switch is thrown. Since CI can be cleaved in the variants, prophage induction should occur, as observed.

Finally, our experiments do not address whether the lytic regulatory state is highly stable in the variants. This state can be highly stable in mutants with a *cIts* allele and mutations blocking lethal lytic functions (Eisen *et al.*, 1970; Calef *et al.*, 1971). In such cases, Cro gains the upper hand at the non-permissive temperature; once it has done so, it continues to repress CI even at the permissive temperature, establishing an 'anti-immune' state. However, with wild-type *cI* and lytic genes, this regulatory state does not need to be highly stable after infection if CI is not expressed. We have not tested whether the anti-immune state is stable in phage with the variant O_R regions; possibly it is not.

Although the variants had qualitatively similar behavior to the wild type, they did display small quantitative differences. Among these, the largest defect was that of λO_R323 in lytic growth. This defect was large enough to allow selective pressure for better growth on plates. Strikingly, all 21 of the large-plaque variants we sequenced proved to have changes in the O_R3 site at the position of O_R1 . Although we have not shown directly that these changes are responsible for improved growth, this finding suggests that changes in this *cis*-acting site are the most frequent way in which the variant overcomes the growth defect.

Among these mutations, the only one that has been analyzed directly for its effect on binding of CI and Cro is the O_R3' mutation (Figure 1B) (Hochschild *et al.*, 1986), which reduces the binding of Cro while slightly strengthening that of CI. We infer that weaker Cro binding is largely responsible for the large-plaque phenotype, and hence that the growth defect of λO_R323 results from inappropriately severe repression of P_R and resulting low levels of lytic gene expression. Although there are several ways this repression could be overcome, the most likely is weakened Cro binding; this cannot readily be achieved by mutations in *cro*, probably because these overproduce a toxic gene product from the P_L promoter (Gussin *et al.*, 1983). In Figure 4 are listed the $\Delta\Delta G$ values measured for these changes in the context of an otherwise wild-type O_R1 site, measured both for Cro (Takeda *et al.*, 1989) and for CI (Sarai and Takeda, 1989), although it is likely that changes in the left half of the operator would not have identical effects in this O_R3 context, since three of the base pairs in this half-site are different. With this caveat, all the changes weaken Cro binding by this criterion. In addition, three of the variants have changes that are likely to affect the strength of P_R . The $-4G \rightarrow A$ and $-4G \rightarrow T$ changes affect the -10 region, the latter probably being an up mutation, while the -8Δ mutation alters the spacing between the -10 and -35 regions, and probably weakens it. The consequence is that P_R is probably weaker but derepressed, so that its pattern of temporal expression should be altered in these particular mutants. It is unclear what would be the consequences for lytic growth.

Most of the pseudorevertants of λO_R323 formed clear plaques, implying that they cannot lysogenize. It is likely that the changes (Figure 4) also reduce the binding of CI

to this site, and the $\Delta\Delta G$ values (Figure 4) are consistent with this expectation. Several variants did form turbid plaques. Among these, $\lambda O_R323'$ and the $+6C \rightarrow T$ variant formed stable lysogens, while lysogens of the $-4G \rightarrow A$ variant formed small colonies and presumably were unstable. This is harder to interpret since this mutation also affects the -10 region of P_R . Finally, two variants formed slightly turbid plaques, but stable lysogens could not be recovered from the plaques; although the $-4G \rightarrow T$ mutation probably weakens CI binding only slightly, it probably increases the strength of P_R , since it changes that position to consensus. In addition, if CI binding to the site at the position of O_R1 were weakened even slightly relative to that when an O_R3 site is at that position, the 3^2 occupancy pattern (Figure 1D) would prevail, blocking stable lysogeny.

Threshold behavior

The threshold behavior of prophage induction is likely to result from cooperative DNA binding by CI to the O_R1 and O_R2 sites, which leads to a steep binding curve for O_R2 (Johnson *et al.*, 1981; Ptashne, 1992). At low UV doses, not enough CI is cleaved to reduce its level to the point that allows P_R expression. The finding that the O_R variants also display threshold behavior is consistent with this explanation. One quantitative difference from the wild type is that the variants had a hair-trigger, in that they induced at lower UV doses than did the wild type (Figure 3). This hair-trigger probably results from multiple causes, including lower levels of CI in all three lysogens, and other causes that differ in the three phages: a full understanding must await further analysis.

The following line of reasoning suggests that this hair-trigger might confer a loss of fitness. Wild-type λ gives efficient induction at approximately the UV dose (10 J/m^2) that begins to kill cells (Bailone *et al.*, 1979; Roberts and Devoret, 1983; Little, 1996). λ has probably evolved to optimize this sensitivity, so that it will not induce at low doses of DNA damage that the cells can survive; hence, a lysogen with a hair-trigger might be less fit. The following evidence suggests, however, that the hair-trigger is not an intrinsic property of the O_R variants. We have isolated derivatives of λO_R323 that efficiently induced only at about a dose of 10 J/m^2 (unpublished data). Several of these carried mutations in the O_R region, while others had changes in cI or outside the cI - O_R - cro interval. These derivatives also showed a threshold response to UV (J.W.Little and K.L.Friedrich, unpublished data). These findings suggest that λO_R323 can evolve to modulate its sensitivity to UV induction. By extension, wild-type λ has probably done so as well.

Evolution of gene regulatory circuitry

Our findings have significant implications for the evolution of gene regulatory circuitry. A long-standing question in evolutionary biology is how complex interlocking circuits such as the λ circuitry can arise. One plausible scenario is that pre-existing simpler circuits become combined by chance, and that a new combination offers a selective advantage, allowing it to become fixed (Campbell and Botstein, 1983).

We suggest that an optimized circuit can evolve in a two-stage pathway. First, a workable initial circuit arises

that functions well enough to offer a selective advantage (in this case, the 'discovery' of a lysogenic lifestyle). Importantly, the robustness we observe ensures that this initial circuit can assume a variety of forms. Secondly, this initial circuit is further refined (or fine tuned) by small changes, as in the $O_R323 \rightarrow O_R323'$ case described here. We predict that this sequential pathway for evolution of regulatory circuits is widespread in biology, although it may be difficult to identify any remnants of the initial circuit.

We recognize that the first step in this pathway is still formidably improbable, but the robustness makes it far more likely, because many possibilities will work. Indeed, it seems likely that this advantage of robust behavior is sufficiently decisive that systems with this property would have been selected for during the course of evolution, for they would be more likely to allow discovery of successful regulatory circuits than would delicately balanced systems (see also Kirschner and Gerhart, 1998). Although a delicate circuit might have a similar likelihood of being fixed, should it arise, the chance of this is far less.

At the same time, robustness is likely not to be simply an evolutionary relic. It offers flexibility to cells, both in tolerance of genetic polymorphisms, particularly in diploid organisms (Hartwell, 1997), and in coping with a wider range of environmental situations (Savageau, 1972). For all these reasons, robust behavior of gene regulatory circuits and other signal transduction systems will probably prove to be the rule rather than the exception.

Materials and methods

Media

Tryptone broth and LB were as described (Miller, 1972) and were supplemented with antibiotics as appropriate. LBGM and LBMM were LB supplemented with 1 mM MgSO_4 and 0.2% glucose or 0.2% maltose, respectively. TMG was 10 mM Tris-HCl , pH 8.0, 10 mM MgSO_4 , $10 \mu\text{g/ml}$ gelatin.

Bacterial strains, plasmids and phages

Most of the strains used are described in Table II.

O_R regions were changed by PCR-based site-directed mutagenesis on plasmids carrying the wild-type cI - O_R - cro interval; $NsiI$ - $BglII$ fragments spanning O_R were then subcloned, along with a synthetic trp attenuator downstream of P_R , into pBS(-) cut with $EcoRI$ and $PstI$, yielding pDWW1 (Figure 1E), pDWW3, pDWW7 and pDWW9, carrying O_R^+ , O_R323 , O_R121 and $O_R3'23'$, respectively. A similar plasmid, pJWL244, carried a $\nu1\nu3$ mutant fragment made by PCR of λvir . For each plasmid, the sequence of the λ -derived segment was identical to that of wild-type λ (DDBJ/EMBL/GenBank accession No. J02459) except for the intended mutations. Details of constructions are available on request.

Construction of variant phages: first, $\lambda \nu1\nu3 kan$ was made as follows: λ^+ was made Kan^R by *in vivo* recombination with the plasmid pE194 (generous gift of Erlan Ramanculov and Ry Young), which carries a segment of λ DNA with a substitution of the non-essential bor gene (which lies beyond R_z at coordinates 46752–46462; Barondess and Beckwith, 1995) by the kan gene of Tn5, followed by infection of JL2497 with the cross progeny and selection of Kan^R lysogens. Phage from these lysogens were termed λkan , and were used to infect cells carrying pJWL244; phage forming clear plaques were isolated from the cross progeny, and their O_R regions were sequenced to identify those with $\nu1\nu3$, yielding $\lambda \nu1\nu3 kan$. Wild-type and variant phages were isolated by *in vivo* recombination of this phage with the plasmids carrying wild-type or altered O_R regions; progeny were screened for turbid plaques, and the sequence of the O_R region was determined. This cross gave approximately one turbid-plaque recombinant per 2000 progeny for wild type. Approximately similar values were seen with progeny of crosses involving the O_R variants, indicating that the initial recombinants did not have to undergo additional mutation to form a turbid plaque.

Table II. Strains used in this work

Strain name	Relevant genotype	Source/reference
Bacterial strains		
JL468	AB1157/F' <i>lacI^q lacZΔM15</i>	Little (1984)
JL573	AB1157(+)	D.Mount
JL783	JL468 Δ(<i>srl-recA</i>)306::Tn10	this work ^a
JL2497	wild type; derivative of N99	Mao and Little (1998)
JL5902	JL2497 Δ(<i>srl-recA</i>)306::Tn10	this work ^b
Phage strains		
λ ⁺	wild type	UV induction of JL573
λ _{vir}	v1 v2 v3	D.Mount
λ _{v1v3 kan}	v1 (in <i>O_R2</i>) v3 (in <i>O_R1</i>)	this work
	<i>bor::kan</i>	
λJL163	<i>O_R⁺ bor::kan</i>	this work
λJL169	<i>O_R121 bor::kan</i>	this work
λJL172	<i>O_R3'23' bor::kan</i>	this work
λJL175	<i>O_R323 bor::kan</i>	this work
Plasmids		
pE194	<i>amp^R bor::kan</i>	E.Ramanculov and R.Young
pJWL244	v1 v3	this work
pDWW1	<i>O_R⁺</i>	this work
pDWW3	<i>O_R323</i>	this work
pDWW7	<i>O_R121</i>	this work
pDWW9	<i>O_R3'23'</i>	this work

^aP1 transduction of JL468 with P1 grown on DM1817 (Little, 1984), followed by selection for resistance to tetracycline.

^bP1 transduction of JL2497 with P1 grown on JL783, followed by selection for resistance to tetracycline.

DNA sequencing

Segments of genomes from various phage strains were amplified by PCR of plaques using the appropriate primers as described (Carlson and Little, 1993), except that PCRs were not asymmetric. PCR products were sequenced by automated cycle sequencing at the Division of Biotechnology, University of Arizona. Plasmids were sequenced directly in the same way.

Isolation of large-plaque variants of λ_{O_R323}

Multiple-plate stocks of this phage were made on JL2497 from single plaques, using fresh tryptone plates and 6 ml of 0.35% top agar. Stocks were plated for single plaques, and variants forming large plaques were purified. From each stock, only one variant of a particular phenotype was chosen.

Biological tests

Lysogens of each phage in strain JL2497 or JL5902 were isolated by kanamycin selection; monolysogens were identified (Powell *et al.*, 1994) and used in all experiments. CI levels: lysogens in JL2497 were grown in LBGM at 37°C; CI levels were assessed by Western blotting, and compared visually with the wild type. In *recA*⁻ hosts, *O_R* variants gave similar levels relative to wild-type as given in Table I (not shown). Burst sizes: JL2497 was grown in LBMM to 2×10⁸/ml, centrifuged and resuspended at 2×10⁹/ml, infected at a multiplicity of infection (m.o.i.) of 0.1 for 20 min at 20°C, and diluted into LBGM. Aliquots were treated with CHCl₃ at intervals to determine the latent period (the time giving one phage/infected cell), which is a measure of the timing of gene expression, and at 90 min for measurement of burst size.

Lysogenization frequency. JL2497 grown in LBMM as above was infected as above at an m.o.i. of 6–8 (a condition that favors the lysogenic pathway). After dilution into LBGM and incubation for 20 min, aliquots were plated in top agar on tryptone plates containing 10 μg/ml kanamycin, and Kan^R colonies were counted.

Free phage. Cultures of lysogens (20 ml for *recA*⁺ strains, 100 ml for *recA*⁻ strains) were grown from a low density to 2×10⁸/ml (*recA*⁺ hosts) or 5×10⁷/ml (*recA*⁻ hosts) in LBGM. Cells were removed by centrifugation followed by CHCl₃ treatment of supernatant fluids, and titers were measured; only turbid plaques were scored.

Prophage induction. Cells were grown in LBGM to 2×10⁸/ml, chilled, centrifuged, resuspended in TMG at 2×10⁸/ml, and irradiated at 254 nm in dim ambient light for various doses at 0.2 J/m²/s. At the indicated doses, aliquots were diluted 10-fold in LBGM, shaken for 2 h at 37°C in the dark, treated with CHCl₃ and titered.

DNase I footprinting

This was carried out as described previously (Carlson and Little, 1993), except that templates were labeled by PCR of the various phages with a 5'-end-labeled primer (Liu and Little, 1998), and were at ~0.2 nM. Incubation with Cro or CI was at 24°C, and binding proceeded for 90 min before addition of DNase I. Purified CI and Cro proteins were the generous gifts of Drs Paul Darling and Gary Ackers.

Analysis of robustness

Robustness (or parameter sensitivity, *S*) is defined as the ratio of the percentage change in an output to the percentage change in a given parameter (Savageau, 1971; Hlavacek and Savageau, 1995). In symbols, $S(X,k) = \partial \log X / \partial \log k \approx \Delta \log X / \Delta \log k = \log(X/X_0) / \log(k/k_0)$, where *X* and *X*₀ are the values of the output in the altered and wild-type systems, and *k* and *k*₀ are the values of the parameter in the altered and wild-type systems, respectively. There are two problems in applying this definition to our system. First, it applies to situations when the behavior changes smoothly in response to parameter changes. In our case, it is plausible that some of the changes we made were large enough that the variants (particularly λ_{O_R323}) were close to the point at which switch-like behavior occurred, i.e. the change in response might be larger than predicted by the equation given above. If so, our data could not be used to obtain the value for *S* at smaller values of *k*. Secondly, in the variant phages we changed not one but two parameters (affinities of Cro and of CI for the altered operator). For this case, the total parameter sensitivity *S*_T(*X*,*k*₁,*k*₂) is the sum of the two individual sensitivities, namely, $S_T(X,k_1,k_2) = S_1(X,k_1) + S_2(X,k_2)$, where subscripts 1 and 2 refer, respectively, to the values for CI and Cro (M.A.Savageau, personal communication). Since we cannot evaluate separately the values of *S*₁ and *S*₂, the overall sensitivities of the system cannot be assessed from our data. More informally, the effects of changes in two parameters could either reinforce or compensate for one another, so that the individual sensitivities could be large or small.

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