Evolution of genetic switch complexity

Gregory W. Broussard and Graham F. Hatfull
Department of Biological Sciences; University of Pittsburgh; Pittsburgh, PA USA

The circuitry of the phage λ genetic switch determining the outcome of lytic or lysogenic growth is well-integrated and complex, raising the question as to how it evolved. It is plausible that it arose from a simpler ancestral switch with fewer components that underwent various additions and refinements, as it adapted to vast numbers of different hosts and conditions. We have recently identified a new class of genetic switches found in mycobacteriophages and other prophages, in which immunity is dependent on integration. These switches contain only three genes (integrase, repressor and cro) and represent a major departure from the λ-like circuitry, lacking many features such as xis, cII and cIII. These small self-contained switches represent an unrealized, elegant circuitry for controlling infection outcome. In this addendum, we propose a model of possible events in the evolution of a complex λ-like switch from a simpler integration-dependent switch.

Introduction

The genetic circuitry controlling the outcome of infection by phage λ is remarkably complex, but presumably arose from an ancestral system that was simpler and had fewer components.1,2 In addition, the genetic switches of other phages that have been studied in some detail show them to be equally complex with many shared and some additional features.3,7 Such complexity could arise from additions and refinements to an ancestral switch that make it work ‘better’ under particular selective pressures.2,8-10 This idea is supported by experimental data showing the non-essential nature of many features of the λ genetic switch and to some extent those of other phages. These include the differential affinities of CI and Cro for O9 operators,11 positive autoregulation of cI,12 cooperativity of CI binding to operator sites,13 negative autoregulation of cII6 and repression of P

PMID:23246436; http://dx.doi.org/10.1016/j.molcel.2012.11.012.

Keywords: genetic switch, genetic circuits, bistable, integration-dependent immunity, lytic and lysogenic growth

Submitted: 02/01/13
Accepted: 03/03/13
http://dx.doi.org/10.4161/bact.24186
Citation: Broussard GW, Hatfull GF. Evolution of genetic switch complexity. Bacteriophage 2013; 3:e24186; http://dx.doi.org/10.4161/bact.24186

Correspondence to: Gregory W. Broussard and Graham F. Hatfull; Email: gwb9@pitt.edu and gfh@pitt.edu

Addendum to: Broussard GW, Oldfield LM, Villanueva VM, Lunt BL, Shine EE, Hatfull GF. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol Cell 2013; 49:237–48; PMID:23246436; http://dx.doi.org/10.1016/j.molcel.2012.11.012.

Addendum to: Broussard GW, Oldfield LM, Villanueva VM, Lunt BL, Shine EE, Hatfull GF. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol Cell 2013; 49:237–48; PMID:23246436; http://dx.doi.org/10.1016/j.molcel.2012.11.012.
direct involvement of integration in deciding the outcome of infection is in contrast to its role in other systems (including λ), where it confers prophage stability, but is not directly involved in determining the outcome of infection. Furthermore, it is attractive as an early evolutionary state, because what biological process is better suited to generate two alternative outcomes than site-specific recombination that yields two mutually exclusive DNA states?

The integration-dependent switches are contained in small ~2 kbp units containing only three genes: integrase (int), repressor (rep) and cro (Fig. 1A); thus, they do not include other components of λ-like switches including xis, cII and cIII. The organization of these genes is such that rep and int are transcribed leftwards, with the putative cro gene transcribed rightwards. Between rep and cro there is a ~200 bp intergenic control region that contains the divergently transcribing promoters P_{rep} (leftwards) and P_{R} (rightwards), and in BPs a 12 bp operator (O_{R}), that overlaps P_{R}, to which repressor binds and down-regulates lytic gene expression (Fig. 1A). Surprisingly, the attP site for integration lies within the rep coding sequence such that different forms of Rep are expressed from viral and prophage genomes (Fig. 1A). This is critical to the function of the switch, since Rep is C-terminally tagged for proteolytic degradation, and integration results in removal of the C-terminal tag and expression of a stable and active form of the repressor. Additionally, Int is also C-terminally tagged for proteolytic degradation rendering it responsive to host cell input signals (proteases such as ClpXP). Integration is thus not only required for establishment of lysogeny, but is the sensor for the central decision-making event in infection. These features are well supported experimentally. For example, a phage mutant with a single amino acid substitution that stabilizes the viral form of the repressor has a much higher frequency of lysogeny than wild-type. Conversely, a phage mutant with a catalytically defective integrase is completely defective in lysogeny.

A rather surprising observation is that Int seems to be able to mediate both integrative and excisive recombination without any apparent requirement for a recombinational directionality factor (RDF). In a plasmid transformation assay in which selection of antibiotic resistance is dependent on integration, transformation is very inefficient because of targeted degradation of Int. But stabilization of Int—which can be accomplished with a single amino acid substitution at its C-terminus—leads to dramatically higher levels of integration and thus higher transformation efficiency of these plasmids. When the stability of the integrated plasmids is examined, we find that transformants generated with the wild-type unstable Int are very stable, and we see no loss after many generations of unselected growth. In contrast, transformants recovered with the stabilized Int show substantial levels of plasmid loss after unselected growth. As the only other phase-encoded component in this assay is repressor, the simplest interpretation is that Int is able to catalyze excisive recombination without need for an RDF. We therefore must assume that prophage stability in a lysogen is largely dependent on low levels of int expression coupled with degradation. This raises the question as to how excision ever occurs. It is plausible that it is simply a stochastic event and that within a given population of cells, there are some that will accumulate sufficient Int to catalyze excision, leading to reconstruction of the inactive viral form of the repressor, and spontaneous induction of lytic growth. An alternative explanation is that expression is induced from adjacent host genes, although no conditions, including mitomycin C exposure, have been identified that lead to efficient prophage induction.

One unresolved question about these integration-dependent immunity systems is how Cro works. We have not been able to delete the putative cro gene, and we presume that it is required for lytic growth. When expressed from a plasmid, it greatly reduces lysogenization frequencies, but does not seem to downregulate the P_{rep} promoter. How Cro works and how repressor synthesis is shut down during lytic growth therefore remain important unanswered questions.

**Evolution of Genetic Switch Complexity**

The compact, self-contained nature of these integration-dependent switches...
Consider the potential impact of a mutation that enhances the expression of $\text{int}$ by forming a new and highly active promoter between $\text{int}$ and $\text{rep}$, $P_i$ (Fig. 1B). This would increase the efficiency of both integrative and excisive recombination, potentially leading to prophage instability under some conditions. Prophage stability makes them attractive as ancestral forms of more complex phage genetic switches. Although they perhaps represent different lineages of switch development from those more closely resembling $\lambda$, they also help to understand potential origins of switch complexity. There are of course numerous possible individual pathways of evolutionary development from one switch type to another, and we will just focus here on some scenarios that could plausibly contribute to development of complex $\lambda$-like switches from the simpler integration-dependent immunity systems.

**Integrase expression and addition of a recombination directionality factor.**
could then be enhanced by a mutation that makes Int dependent on a host protein for excision, perhaps through protein-protein interactions (Fig. 1B). This gene could then be acquired by the phage to act as the recombination directionality factor (RDF), followed by subsequent acquisition of an additional DNA binding domain to Int, and arm-type binding sites to attP such that recombination is now regulated by the DNA binding properties of the RDF (Fig. 1B). A scenario of this type is supported by the observation that serine-integrases use simple sites that lack the equivalent of arm-type binding sites in the lambda system, and the RDF acts through direct subunit-subunit interactions with Int. Several RDFs of serine-integrases have been described that are unrelated at the sequence level, but these include relatives of the family that includes lambda Xis. At least in one example, this RDF is capable of DNA binding even though DNA binding is not required for its action. These thus represent plausible examples of intermediate stages in the evolution of tyrosine integrase systems typified by phage lambda.

**Relocation of int and attP.** The events described above—including separation of int from the P promoter—could be facilitated by a duplication event that inserts a second copy of the attP-int cassette elsewhere in the phage genome (in lambda, int is separated by over 8 kbp from cl) (Fig. 1C). Such a rearrangement could itself be mediated by aberrant Int-mediated recombination, and we note that there is at least one example of an apparent re-location of an integration cassette in mycobacteriophage Giles. Such a duplication event is attractive because it would enable the further evolution of the newly added cassette, and development of a repressor system that is independent of recombination as a central control mechanism. Furthermore, with addition of a RDF and mechanistic separation of integration and excision events, these would be less responsive to the integrase concentrations, and reduce dependency on the rate of degradation of Int from its C-terminal tag, leading to its loss (Fig. 1C). However, this would also demand an alternative sensor for determining the outcomes of infection, which would then require an alternative means of regulating repressor expression.

**Regulation of repressor and separation of lysogeny establishment and maintenance functions.** With loss of dependence on integration for immunity, other processes for control of repressor expression must be acquired, as well as a separation of mechanisms for expressing repressor for lysogenic establishment, as opposed to maintenance. One scenario is to imagine a circumstance in which the ancestral proteolytically tagged repressor regained function by the ability to bind to RNA Polymerase and activate its own expression (Fig. 1C). This is attractive, as we have demonstrated that overexpression of the viral form of the BP repressor at least partially restores immunity. This could then lead to loss of the C-terminal proteolytic tag on repressor, as this would no longer serve a critical role (Fig. 1C). However, loss of the tag generating a stable form of the repressor that activates its own expression could give rise to runaway autoactivation and accumulation of very high levels of repressor in the cell. An important consequence of this is that spontaneous induction of lytic growth would now be reduced to very low levels, and as virtually all temperate phages undergo readily detectable levels of spontaneous induction, we assume that this is a biologically important event.

The selective pressures to downregulate repressor synthesis could involve numerous mechanisms, but an attractive one is the introduction of mutations that now make repressor synthesis dependent on autoactivation (Fig. 1C). This phage would grow fine lytically, but be capable of only very low lysogenization frequencies, although the lysogens formed would be quite stable. Elevation back to normal lysogenization frequencies could be accomplished by mutations that generate a promoter (equivalent to P) that is activated by a host transcriptional activator followed by acquisition of that activator from the host (equivalent to cl) (Fig. 1C). This activator could later gain control of the P promoter driving int expression (Fig. 1C). Negative autoregulation and cooperativity of repressor binding could be added to further fine-tune the switch (Fig. 1C).

**Putting the pieces together.** Combined into one genomic context, the above-mentioned refinements would make up a genetic circuitry resembling the λ switch in many respects (Fig. 1C). Because of the mosaic nature of phage genomes and the prevalence of recombination within and between genomes, evolutionary events leading to complex genetic circuits are unlikely to have occurred in a single genomic context. It seems more likely that events would take place within a large variety of different phage genomes with different hosts, but in a scenario in which all phage genomes have access to a large common gene pool, albeit at different frequencies. There would be plenty of opportunity for these components to find themselves in common genomes, especially given the vast scale and dynamic nature of the phage population.

**Concluding Remarks**

Because of the prevalence of the temperate lifestyle among phages and their vast diversity, it is likely that as more phage genomes are sequenced, we will gain a greatly improved understanding of the evolution of genetic switches. At this point, we have very few examples of the variety of genetic switches in existence and the circuitry of most have only been studied to a partial level. Furthermore, we have a limited knowledge of the biology of phages in the context of selective pressures phages encounter outside of a laboratory setting. However, as our knowledge of the ecology of phages and the types of natural genetic switches expands, and as efforts to engineer synthetic genetic switches leads to new insights, our understanding of how complex genetic circuits evolve and the variety of circuitries possible will be greatly enhanced. This will further allow us to control gene expression to a level of sophistication that will open many possibilities for research, medical and industrial use of finely tuned genetic switches.

**Disclosure of Potential Conflicts of Interest**

The authors verify that there is no conflict of interest concerning this manuscript.

**Acknowledgments**

We thank Valerie Villanueva and Lauren Oldfield for comments on the manuscript.
This work was supported by NIH grants GM093901 and AI059114.

References

1. Court DL, Oppenheim AB, Adhya SL. A new look at bacteriophage lambda genetic networks. J Bacteriol 2007; 189:298-304; PMID:17085553; http://dx.doi.org/10.1128/JB.01215-06.

2. Little JW. Evolution of complex gene regulatory circuits by addition of refinements. Curr Biol 2010; 20:R724-34; PMID:20833317; http://dx.doi.org/10.1016/j.cub.2010.06.028.

3. Campbell A. Comparative molecular biology of lambda phages. Annu Rev Microbiol 1994; 48:193-222; PMID:7826095; http://dx.doi.org/10.1146/annurev.mi.48.100194.001205.

4. Knight KL, Bowie JU, Vershon AK, Kelley RD, Smith MC. A phage protein that binds φ22 DNA to prevent its replication by bacteriophage 22. Microbiol Rev 1978; 42:385-413; PMID:353481.

5. Krause HM, Higgins NP. Positive and negative regulation of the Mu operator by Mu repressor and Escherichia coli integration host factor. J Biol Chem 1999; 274:15849-55; PMID:10538469; http://dx.doi.org/10.1074/jbc.274.24.15849.

6. Goss P, Wain LR, Harfalk GF. Control of phage Bb1 excision by a novel recombination directionality factor. PLoS Biol 2006; 4:e186; PMID:16719562; http://dx.doi.org/10.1371/journal.pbio.0040186.

7. Khaleel T, Younger E, McEwan AR, Varghese AS, Smith MC. A phage protein that binds φ22 DNA integrates to switch its directionality. Mol Microbiol 2011; 80:1450-63; PMID:21564337; http://dx.doi.org/10.1111/j.1365-2958.2011.07963.x.

8. Bhattacharyya RP, Reményi A, Egan JB, Harfalk GF. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol Cell 2011; 49:237-48; PMID:23246436; http://dx.doi.org/10.1016/j.molcel.2011.11.012.

9. Jacob F. Evolution and tinkering. Science 1977; 196:1161-6; PMID:860134; http://dx.doi.org/10.1126/science.860134.

10. Patshnev M, Gann A. Imposing specificity by localisation: mechanism and evolvability. Curr Biol 1998; 8:R897; PMID:9844213.

11. Little JW, Shepley DP, Wert DW. Robustness of a gene regulatory circuit. EMBO J 1999; 18:4299-307; PMID:10428968; http://dx.doi.org/10.1093/emboj/18.15.4299.

12. Michalek CB, Little JW. Positive autoregulation of cl is a dispensable feature of the phage lambda gene regulatory circuitry. J Bacteriol 2005; 187:6430-42; PMID:16159777; http://dx.doi.org/10.1128/JB.187.18.6430-6442.2005.