

# Contrasting lifestyles of rolling-circle phages and plasmids

Richard P. Novick

The rolling-circle mechanism of DNA replication is used by small prokaryotic genomes, such as single-stranded phages and plasmids. However, phages and plasmids have adapted the rolling-circle mechanism differently to suit their contrasting biological needs. The  $\phi$ X174 phage uses a monomeric initiator protein catalytically, displays incomplete termination and recycles the initiator protein, in order to mass-produce phage progeny. By contrast, to control replication precisely, the pT181 plasmid uses a dimeric initiator protein stoichiometrically, completes termination and inactivates the initiator after each replication cycle. The  $\phi$ X174 phage and the pT181 plasmid represent paradigmatic adaptations of the rolling-circle mechanism and could provide models for other replicons.

**SMALL PROKARYOTIC GENOMES** replicate by the rolling-circle (RC) mechanism. Subtle adaptations of the RC paradigm are evident in phage and plasmids (Fig. 1), and are a consequence of the contrasting biological needs of these replicons. Phage progeny are mass-produced on an assembly line and therefore efficiently utilize the cell's molecular resources to produce the largest possible number of progeny before lysis. Plasmid molecules, by contrast, must maintain a stable hereditary relationship with the host and are therefore individually crafted in a precisely controlled process. Here, I review the mechanism of RC replication and discuss the adaptations of the RC that are evident in the  $\phi$ X174 phage and the pT181 plasmid, and other replicons that use the RC mechanism.

## Rolling-circle replication

All naturally occurring RC replicons encode replicon-specific initiator proteins that belong to the strand-transferase superfamily<sup>1</sup>. These proteins initiate replication by binding to a specific site in the leading-strand replication origin and introducing a sequence-specific nick at a nearby site<sup>2</sup>. A tyrosine residue in the protein's active site is central to nick formation; in some, but not all, cases, the initiator remains attached, through a phosphotyrosine bond, to the 5' nucleotide at the nick site. RC initiators prefer

ssDNA substrates, which they generate by melting the nick site, but only if the substrate is supercoiled. RC initiators generally contain two functional tyrosine residues; at least one tyrosine residue therefore remains unbound during the replication cycle. Following, or in concert with, the introduction of the nick, replication proteins assemble to form a replisome, which includes ssDNA-binding protein (SSB), polymerase III holoenzyme and Rep helicase, as well as the initiator.

Polymerization proceeds by a simple strand displacement, using the 3' OH generated at the nick site as a primer. The bound initiator protein travels with the replication fork, generating a looped RC (Ref. 2). As soon as the replication fork leaves the vicinity of the leading-strand origin, it exposes a new copy of the nick site; this new copy comprises the junction between the 3' end of the old leading strand (shown in green in Fig. 1c) and the 5' end of the new strand (shown in red in Fig. 1c). The junction must not be susceptible to secondary nicking by the initiator, because secondary nicking would disrupt the replication cycle; this probably explains why the initiation of RC replication requires a supercoiled molecule – that is, the ability of the initiator to melt its target site is carefully determined to be so weak that it requires an assist from the free energy of supercoiling plus the palindromic secondary structure surrounding the nick site<sup>3-5</sup>. Because the replicating molecule is relaxed, the newly exposed nick site cannot be melted and nicked.

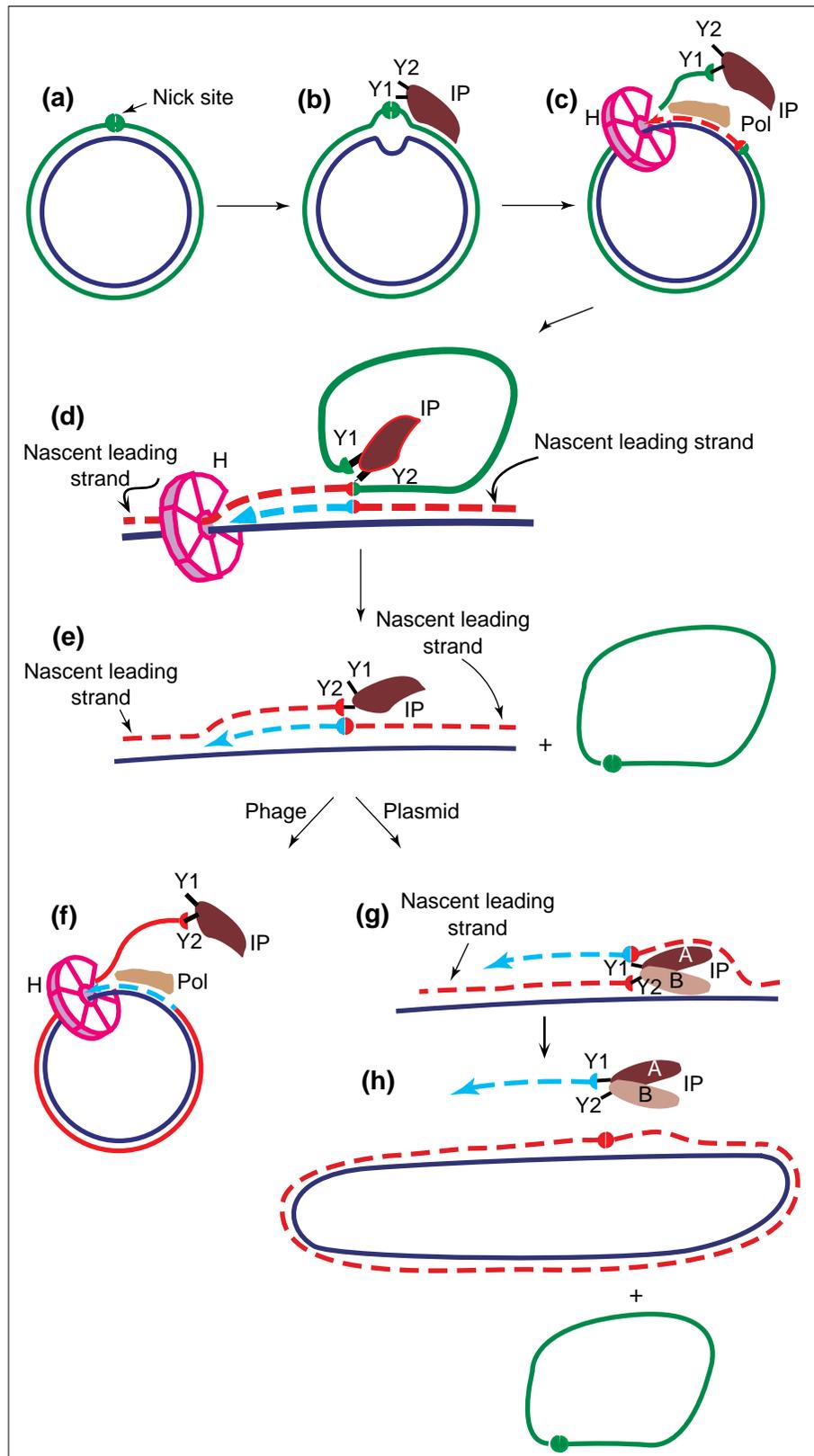
Termination occurs when the replication fork returns to the double-strand origin and requires the participation of the initiator protein (almost certainly the same molecule that was used for initiation). The nascent leading strand is extended for a short distance past the initiation nick site; at this point, the replication fork probably pauses, possibly owing to release of the bound helicase. The short 3' extension of the leading strand (shown in lighter blue in Fig. 1d) displaces the junction between old and nascent leading strands. This junction, which corresponds to the initiator-recognition site and is now single stranded, is then cleaved by the initiator, in a reaction that involves the second active-site tyrosine residue. A transesterification reaction occurs next: the newly released 3' end of the old leading strand is transferred to the 5' end of the same strand. This recircularizes the old leading strand, which is released from the replisome as a single-stranded circle (Fig. 1e). At this point, there is a critical difference between phage and plasmid.

**Phages.** Termination is incomplete: following release of the recircularized leading strand, the protein attached (through the second tyrosine residue) to the 5' end of the nascent leading strand starts a new cycle of polymerization, possibly by re-binding to the Rep helicase (Fig. 1f). This is referred to as re-initiation – which is something of a misnomer because leading-strand extension is only paused, not interrupted. Theoretically, this recycling could, by alternating between the two tyrosine residues<sup>6,7</sup>, continue indefinitely; in practice, it usually continues for ~20 cycles. When ~35 double-stranded molecules have accumulated, lagging-strand replication stops, and new progeny plus strands are encapsidated to form progeny phage particles. This switch is brought about by the accumulation of capsomeres and phage protein C, and seems to be carefully timed to exhaust host-cell resources for the production of progeny phage before cell lysis. Phage protein C is required for the packaging reaction and also reduces the rate of *de novo* initiation on double-stranded templates – thus coordinating the synthesis and encapsidation of plus strands<sup>8</sup>. In the case of  $\phi$ X174, the initiator has not been shown to melt the region surrounding the nick site. Two important questions therefore remain: do all RC phage initiators actively melt the double-stranded origin, and what is responsible for the pausing and resumption of replication between cycles?

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**Plasmids.** RepC, the initiator encoded by the pT181 plasmid contains only a single active-site tyrosine residue but is dimeric (RepC–RepC); thus, each functional unit also contains two active tyrosine residues – either of which can be used for initiation. Termination of plasmid DNA replication (Fig. 1g,h) is completed by a second transesterification, in which the new leading strand is circularized, remaining paired with its template. The initiator is released with the oligonucleotide that corresponds to the short 3' leading-strand extension, which is attached to one subunit, as a RepC–RepC\* heterodimer<sup>9</sup>. The second transesterification probably occurs following spontaneous (reversible) displacement of the extended 3' end of the nascent leading strand by its 5' end, which generates the single-stranded substrate required for cleavage (Fig. 1g)<sup>5</sup>.

The heterodimeric initiator protein, RepC–RepC\*, lacks initiating activity<sup>9</sup>. Thus, the second transesterification not only completes the termination process but also inactivates the initiator protein. Consequently, each replication event generates exactly two progeny molecules and ensures that replication cannot be re-initiated until some later point, when the entire process must start *de novo*, using a newly synthesized initiator dimer. This second stage thus represents the critical difference between pT181 and  $\phi$ X174: the phage DNA recycles the initiator protein and continues replicating, whereas the plasmid completes



**Figure 1**

Models for rolling circle replication of  $\phi$ X174 and pT181 (as models for phage and plasmids, respectively). **(a)** Double-stranded form of the replicon. **(b)** The initiator protein (IP), which contains the two active-site tyrosine residues (Y1 and Y2), binds to and melts the region surrounding the nick site. **(c)** After assembly of the replisome, the 3' extension of the leading strand begins (shown in red). The IP is attached by Y1 to the 5' end of the displaced leading strand (shown in green). The displaced leading strand would be coated by ssDNA-binding protein. **(d)** After the replication fork has completed a cycle of replication, it pauses, having extended the leading strand for a short distance past the nick site. This extension (shown in lighter blue) displaces the junction between the old (shown in green) and nascent (shown in red) leading strands. Y2 then cleaves this junction. **(e)** Transesterification (attack of the phosphotyrosine bond between Y1 and the 5' end of leading strand by the newly released 3' end of the displaced leading) displaces the IP, and the leading strand recircularizes and is released as a single-stranded circle (shown in green). The IP is now attached by Y2 to the 5' end of the nascent leading strand. In this model, the helicase and polymerase have been released from the complex. Steps (a–e) are common to both  $\phi$ X174 and pT181. **(f)** In  $\phi$ X174, the replication complex is re-assembled and 3' extension of the leading strand resumes. This step is identical to (c), except that the IP is attached to the displaced leading strand by Y2 instead of Y1. **(g)** In pT181, the 5' end of the new leading strand, which is attached to the IP through Y2 (of subunit B), displaces its own extended 3' end, which allows cleavage by Y1 (of subunit A). **(h)** Following cleavage, the free 3' end of the new leading strand attacks the Y2–DNA bond; this causes transesterification, which circularizes the leading strand and releases the IP, which is bound to the oligonucleotide that is the 3' extension of the new leading strand (lighter blue) through Y1. H, Rep helicase; Pol, polymerase III holoenzyme.

the termination process and inactivates the initiator. The factor(s) responsible for this difference remain to be identified.

**Lagging-strand replication.** With both plasmid and phage, lagging-strand replication occurs subsequent to and independently of leading-strand replication, using a different sequence as an origin, and does not involve the initiator protein. Rather, it uses an RNA primer (synthesized by one of several possible mechanisms) plus SSB, Pol III and a variable set of other replication proteins that differs among different systems. Termination presumably occurs by simple ligation and generates a relaxed CCC monomer, which is then acted upon by gyrase plus ATP to restore the native superhelicity. Note that the lagging-strand-replication mechanism used by  $\phi$ X174 is presumably the same as that used to synthesize the complementary minus strand following phage infection.

### Regulation

The differing lifestyle requirements of typical plasmids (e.g. pT181) and phage (e.g.  $\phi$ X174) dictate that the plasmid must coordinate its replication with that of the host cell – so as to maintain a constant copy number – whereas the phage is set up to produce as many progeny as possible during the lytic cycle.

Generally, plasmid replication-control systems regulate the rate of initiator synthesis; in order for this to determine the copy number, the initiator must be used stoichiometrically rather than catalytically – a second critical difference between the phage and plasmid lifestyles. Thus, for pT181, and presumably for other RC plasmid systems, one initiator molecule (dimer) is synthesized per replication event<sup>10</sup>, and inactivation of the initiator at the end of the replication cycle automatically ensures stoichiometry<sup>9</sup>. Similarly, for ColE1 and other members of its family, the initiator is an RNA primer; synthesis of the primer initiates a replication cycle, and the primer is degraded automatically after use. For RC plasmids, as well as members of the ColE1 family, initiator synthesis is controlled by antisense RNAs<sup>11</sup>, sometimes, with the addition of repressor proteins<sup>12</sup>. For  $\phi$ X174, reproduction is regulated by switching from exponential re-duplication of the double-stranded form to linear production of single-stranded progeny molecules – when enough of the former have been produced to ensure that the maximum number of phage particles will be generated before cell lysis.

### The inactivated pT181 initiator

Attachment of an oligonucleotide to RepC–RepC, which is the final event in the termination of pT181 replication, generates a heterodimer, RepC–RepC\*, that has one derivatized subunit (covalently linked to the oligonucleotide) and one native subunit. The properties of this heterodimer are subtly different from those of the native RepC–RepC homodimer. Recall that the pT181 leading-strand origin contains two adjacent inverted repeats (IRs): IRII, which contains the nick site at its tip; and IRIII, which constitutes the primary recognition/binding site for the initiator<sup>13,14</sup>. Although the RepC–RepC\* heterodimer can bind IRIII, this binding is four times weaker than that of RepC–RepC; RepC–RepC\* cannot melt the IR-II region and cannot initiate replication, despite having a free active-site tyrosine residue on one subunit<sup>15</sup>. This confirms the hypothesis that a plasmid initiator must be used stoichiometrically; the initiators of all six pT181-family plasmids, and possibly pUB110, undergo this (tyrosine) modification<sup>16,17</sup>. Nordstrom and co-workers<sup>18</sup> observed long ago that the initiator protein of the theta plasmid R1 must be synthesized *de novo* for each replication event, which is consistent with stoichiometric use of the initiator.

The free active-site tyrosine residue of the modified pT181 initiator heterodimer is fully functional: it can nick and relax a supercoiled substrate that contains the pT181-leading-strand replication origin<sup>15</sup>, and it can catalyze strand-transfer reactions that involve oligonucleotides that contain its recognition site as efficiently as the native RepC–RepC homodimer<sup>5</sup>. RepC–RepC\* nicking–relaxing activity, however, is much weaker than that of the native homodimer and, unlike that of the native homodimer, is extremely cold sensitive. This suggests that RepC–RepC\* can nick only those molecules in which the IR-II palindromic element has spontaneously extruded to form a cruciform<sup>3,5</sup> – a process driven by the free energy of supercoiling.

Although the presence of the oligonucleotide would obviously disable the termination function of the protein, it is not clear why RepC–RepC\* cannot initiate. One possibility is that a RepC dimer that has both active-site tyrosine residues derivatized cannot bind the PcrA helicase, because the attached DNA induces an allosteric modification in the protein's structure. As shown in Fig. 2c, nicking by RepC–RepC\* would

generate a DNA–protein complex in which the protein is bound to the 5' side of the nick and therefore has both tyrosines derivatized. This could also explain why replication halts during termination: the helicase would be released as soon as the first cleavage occurs, because this would also generate an initiator derivative in which both tyrosine residues were bound to DNA (see Fig. 1d). Interestingly, overproduction of RepC leads to a decrease in replication, irreversible loss of supercoiled plasmid DNA *in vivo*<sup>19</sup> and accumulation of a much greater amount of RepC–RepC\* than can be accounted for by replication (R. Jin and R. Novick, unpublished). This inhibition could be accounted for by the scheme shown in Fig. 2. Although the structure in Fig. 2c cannot replicate, it can resolve by transesterification, generating either the structure in Fig. 2d or the structure in Fig. 2e; alternatively, it could undergo abortive initiation (the structure in Fig. 2f), which would lead to RepC–RepC\* overproduction.

I suggest that oligonucleotide attachment is a very convenient means of inactivating the initiator and is built into the termination mechanism, but that the plasmid pays a high price for this convenience: the derivatized initiator could interfere with normal replication, because it is present at a concentration at least ten times higher than that of the active homodimer and is metabolically stable<sup>9</sup>. We have observed inhibition of the normal functions of the native homodimer (including replication) by the heterodimer *in vitro*, at concentrations similar to those seen *in vivo*<sup>20</sup>. Significantly, during normal growth, only a very small proportion of pT181 DNA is in the nicked or relaxed form at any given time point, even with very-high-copy-number plasmids, despite the very high levels of RepC–RepC\* heterodimers present. Perhaps the level of unrestrained supercoiling *in vivo* is insufficient to support spontaneous extrusion of the IRII cruciform at an appreciable frequency – especially given that this cruciform is not the most stable secondary structure in the plasmid<sup>3</sup>.

### Initiation and termination in filamentous single-stranded phages

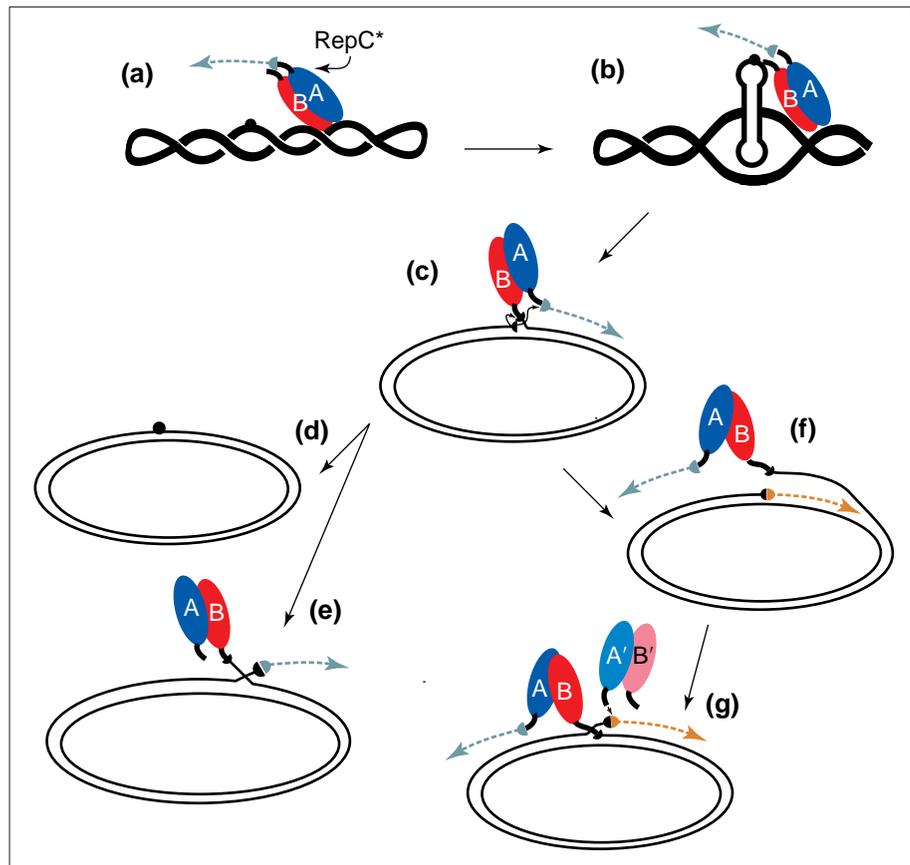
Filamentous single-stranded phages are intermediates between lytic phages (e.g.  $\phi$ X174) and plasmids (e.g. pT181): they do not cause cell lysis but exist intracellularly as stable plasmids and generate infective particles more or less indefinitely. This intermediate biotype is

reflected in the termination mechanism. The displaced leading strand is circularized and released as a single-stranded monomer, whereas the nascent leading strand remains open, as in  $\phi$ X174, permitting resumption of replication. Note that the nick site in the single-stranded filamentous phage fd is homologous to that of pT181 and includes an IR that has the potential to form a hairpin flanking the nick site.

The initiator, Gp2, actively melts and nicks the double-strand origin; the intact IR is not required for nicking<sup>4</sup>, although it seems to be required for replication<sup>21</sup>. However, the precise sequence of events that occurs between replication cycles is unclear. The initiator probably remains associated with the origin and supports resumption of leading-strand extension as soon as the displaced leading strand has been released, although each replicating molecule appears to be cycled only two or three times (T. Meyer, pers. commun.) – rather than 20 or more, as with  $\phi$ X174. Presumably, the nascent leading strand is circularized with a certain probability, and the resulting double-stranded circle could then be used for *de novo* initiation only after having been wound up by gyrase.

A significant difference between  $\phi$ X174 and fd is the fact that the fd initiator, Gp2, functions as a tetramer and is not covalently attached to the DNA during replication, although it travels with the fork and is an essential component of the replisome. Similarly, the pMV158-plasmid initiator, RepB, also does not form a covalent link with the leading strand and appears to function as a hexamer<sup>22</sup>. Because the fd-infected cell is not lysed but continues to produce infective phage particles, it represents a steady state in which the copy number of the double-stranded circles and the rate of production of phage particles are relatively constant.

Following infection, plasmid-like multiplication proceeds until the population of double-stranded circles reaches a certain size (several hundred), at which point the rate of replication is balanced by the rate of formation of infective phage particles, as well as by growth of the bacteria. One determinant of this balance is the concentration of the gene X product, a 13-kD protein encoded in the C-terminal region of gene II. This protein is required for accumulation of plus-strand monomers and inhibits plasmid-like replication<sup>23</sup>; it is thus analogous to the product of  $\phi$ X174 gene C.



**Figure 2**

Model for nicking of supercoiled DNA by RepC–RepC\*. (a) RepC–RepC\* binds to its recognition site on supercoiled pT181 DNA. (b) The cruciform is spontaneously extruded. (c) Subunit B nicks the DNA and becomes attached. (d) Trans-esterification involving the free 3' end and subunit B reverses the nicking reaction, giving rise to a relaxed covalently closed circular monomer. (e) Trans-esterification involving subunit A generates a form in which subunit A remains attached and the oligonucleotide is transferred to the 5' end of the plus strand<sup>5</sup>. (f) The free 3' end of the nicked strand is extended by a few nucleotides, displaying the 5' end attached to subunit B (brown dashed arrow). (g) The newly extended 3' end is displaced by its homolog attached to subunit B. This renders the new nick site single stranded. A new molecule of RepC–RepC\* can now cleave this junction and generate a new molecule of RepC–RepC\* without replication.

The concentration of the gene 5 product, a ssDNA-binding protein that sequesters the progeny single-stranded monomers, must also be important. No RC plasmid is known to encode either of these functions, although the host cell's SSB is always required.

#### Other rolling-circle plasmids

In RC plasmids of the pE194–pMV158 family, the initiation nick site is flanked by a GC-rich IR; however, we do not know whether or not this IR is extruded as a cruciform. We also do not know whether initiator recycling is prevented or, for example, whether an inactive derivative is formed.

In the case of the pUB110 plasmid, a member of the pC194 family, a derivative of its initiator, RepU, accumulates. This derivative has a decreased electrophoretic mobility reminiscent of that seen for RepC–RepC\* (Ref. 17). However, the

replication origin contains only a rather weak IR, which is not especially GC-rich. Noirot-Gros and Ehrlich have proposed that the prototypical member of this family, pC194, terminates replication by an entirely different mechanism, which involves a transesterification initiated by a highly conserved glutamate residue four residues away from the putative active-site tyrosine residue<sup>24</sup>. Such a termination mechanism would release the protein in an unmodified form after completion of the two stages of termination necessary for plasmids. Thus, it is again not clear whether a specific mechanism prevents recycling of the initiator.

Unfortunately, we do not yet have sufficient information about other RC plasmids to make generalizations about the mechanism of termination or address the question of whether recycling of the initiator is prevented (and, if so, how it is prevented). Although the prototypical

RC plasmid (pT181) and phage ( $\phi$ X174) represent very well-defined biotypes, with respect to their functional strategies, we therefore cannot predict how generally their adaptations of the rolling-circle mechanism apply.

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## Harnessing free radicals: formation and function of the tyrosyl radical in ribonucleotide reductase

JoAnne Stubbe and Pam Riggs-Gelasco

Ribonucleotide reductases (RNRs) are uniquely responsible for converting nucleotides to deoxynucleotides in all organisms. The cofactor of class-I RNRs comprises a di-iron cluster and a tyrosyl radical, and is essential for initiation of radical-dependent nucleotide reduction. Recently, much progress has been made in understanding the mechanism by which this cofactor is generated *in vitro* and *in vivo*, as well as the function of the tyrosyl radical in nucleotide reduction. The *Escherichia coli* RNR cofactor provides a paradigm for cofactors in other di-iron requiring or tyrosyl-radical-requiring proteins.

**THE FRONT PAGES** of many newspapers describe the scourge of free radicals, their ability to destroy themselves and their surroundings. Why is it, therefore, that nature has chosen these uncontrollable free radicals as essential components of central steps in metabolism? A system that functions as a paradigm for exquisite control of radicals is the ribonucleotide reductases (RNRs). In all organisms, these enzymes catalyze the conversion of nucleotides to deoxynucleotides, providing the monomeric precursors required for DNA biosynthesis. Despite their central role in deoxynucleotide formation,

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RNRs from various organisms are distinctly different and have been divided into classes on the basis of their cofactor requirements (Fig. 1). Each metallocofactor is proposed to function as a radical-chain initiator to generate a transient thiyl radical,  $\cdot$ Cys (the radical is located on a cysteine residue within the protein), that initiates nucleotide reduction.

Here, we focus on the class-I RNRs, for which the *Escherichia coli* enzyme serves as a prototype. These RNRs comprise two homodimeric subunits: R1 and R2. R1 is the center where the complex nucleotide-reduction process occurs, and the recently determined crystal structure of this subunit<sup>1</sup> serves as a paradigm for the secondary and tertiary structures adopted by the active sites of all classes of RNR (Ref. 2). R2 has also been examined crystallographically<sup>3,4</sup> and contains the diiron(III)-tyrosyl-radical

( $\cdot$ Tyr) cofactor discovered by Sjöberg *et al.*<sup>5</sup> The  $\cdot$ Tyr has unusual chemical stability ( $t_{1/2} = 4$  days, for the  $\cdot$ Tyr in R2 – compared to a  $t_{1/2}$  of milliseconds, for the  $\cdot$ Tyr in solution) and is essential to the nucleotide-reduction process. The class-I-RNR diiron metal cluster and amino-acid free radical provide a model for the mechanism of metal-cofactor assembly and the basis for the differential chemical reactivities of a wide range of proteins that require similar diiron clusters for catalysis. Here, we address two questions. (1) How are the essential  $\cdot$ Tyr and diiron(III) cluster of the class-I RNRs formed? Is the diiron center representative of cofactor assembly and function in other diiron requiring proteins? (2) What is the function of this  $\cdot$ Tyr in the nucleotide-reduction process? Is it representative of the role of  $\cdot$ Tyr in other enzymes?

#### Formation of the diiron(III)-tyrosyl-radical cofactor of class-I RNRs

***In vitro* studies.** In the past few years, we have learned much about the assembly, *in vitro*, of both the diiron(III)- $\cdot$ Tyr cofactor from apo-R2 (R2 without metal bound)<sup>6</sup> and the diiron(II) R2 (R2 in which each iron in the diiron site is in the +2 oxidation state). High-resolution structures of apo-R2, diiron(II) R2 and diiron(III) R2 are now available<sup>3,4,6</sup> (Fig. 2). The early studies of Atkin *et al.*<sup>7</sup> have facilitated a variety of time-resolved physical-biochemical studies on the cofactor-assembly process<sup>8–10</sup>. Together with similar studies on a second dinuclear-iron-requiring enzyme, methane monooxygenase<sup>11,12</sup>, and on several mononuclear non-heme iron enzymes<sup>13</sup>, several themes are emerging.

First, as presaged by model studies by the Lippard laboratory<sup>14</sup>, ligand reorganization plays an essential role in