semaphorins (*26*, *27*). Given the presence of all three types of molecules in the SA library, it is likely that semaphorins play a role in stem cell adhesion and homing behavior.

Our studies identify numerous individual candidate regulatory molecules, but they also pave the way for more global approaches to stem cell biology. In particular, the production of stem cell microarrays will permit the analysis of fluctuations in the genetic program as a function of permutations in self-renewal, commitment, or other stem cell properties (*3*, *4*, *28*). The large collection of gene products will also facilitate proteomic strategies to uncover protein interaction networks (*29*). We anticipate that the SCDb will be a resource for the stem cell community and will foster the collaborative and consortial interactions necessary for global approaches to important biological questions.

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Role of 4.5*S* RNA in Assembly of the Bacterial Signal Recognition Particle with Its Receptor

Paul Peluso,[1] Daniel Herschlag,[2] Silke Nock,[1] Douglas M. Freymann,* Arthur E. Johnson,[3] Peter Walter[1]†

The mechanism by which a signal recognition particle (SRP) and its receptor mediate protein targeting to the endoplasmic reticulum or to the bacterial plasma membrane is evolutionarily conserved. In *Escherichia coli*, this reaction is mediated by the Ffh/4.5*S* RNA ribonucleoprotein complex (Ffh/4.5*S* RNP; the SRP) and the FtsY protein (the SRP receptor). We have quantified the effects of 4.5*S* RNA on Ffh-FtsY complex formation by monitoring changes in tryptophan fluorescence. Surprisingly, 4.5*S* RNA facilitates both assembly and disassembly of the Ffh-FtsY complex to a similar extent. These results provide an example of an RNA molecule facilitating protein-protein interactions in a catalytic fashion.

Ffh and FtsY are both guanosine triphosphatases (GTPases) (*1*–*5*) that interact with each other in a GTP-dependent manner and reciprocally stimulate each other's GTPase activity (*6*, *7*). The GTPase domains of Ffh and FtsY define them as members of a GTPase subfamily with unique properties (*8*–*11*). 4.5*S* RNA enhances association of Ffh and FtsY, which suggested a role for the RNA in stabilizing the complex (*6*). To analyze the role of 4.5*S* RNA in this reaction in more detail, we took advantage of the fact that FtsY, but not Ffh, contains tryptophan residues. This allowed us to monitor the interaction of Ffh and FtsY spectroscopically (Fig. 1). Recently, a similar assay was independently developed by Jagath and co-workers (*12*). In our studies we used a NH_2 terminally truncated version of FtsY (residues 48 to 494) that was previously shown to interact with Ffh in a manner indistinguishable from that of full-length FtsY (*5*).

Incubation of FtsY with Ffh/4.5*S* RNP in the presence of the nonhydrolyzable GTP analog GppNHp (5′-guanylylimidodiphosphate) shifted the tryptophan fluorescence emission maximum by ~10 nm and doubled the fluorescence intensity (Fig. 1A). This is consistent with burial of one or both of the tryptophans in a more hydrophobic environment upon formation of a Ffh-FtsY complex. These fluorescence changes occurred only in the presence of GppNHp and not in the presence of guanosine diphosphate (GDP) (Fig. 1B), consistent with the GppNHp dependence for complex formation determined by affinity chromatography (*6*).

Complex formation and stimulation of GTPase activity were previously shown to be dependent on the presence of 4.5*S* RNA. We were therefore surprised to observe that in the absence of 4.5*S* RNA, addition of GppNHp resulted in an increase and shift in fluorescence that was indistinguishable from that observed with the Ffh/4.5*S* RNP (Fig. 1, C and D). To understand the origin of this paradox and the role of 4.5*S* RNA in complex formation, we carried out a kinetic and thermodynamic analysis of the reaction.

We compared the kinetics of association between Ffh and FtsY in the absence and presence of 4.5*S* RNA (Fig. 2, A and B). The association with FtsY, monitored by fluorescence, was faster for Ffh/4.5*S* RNP than for the same concentration of Ffh by a factor of more than 100. Analogous determinations at a series of Ffh and Ffh/4.5*S* RNP concentrations gave second-order rate constants for

[1]Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA. [2]Department of Biochemistry, Stanford University, Stanford, CA 94305, USA. [3]System Health Science Center, Texas A&M University, College Station, TX 77843, USA.

*Present address: Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611, USA.

†To whom correspondence should be addressed. E-mail: walter@cgl.ucsf.edu

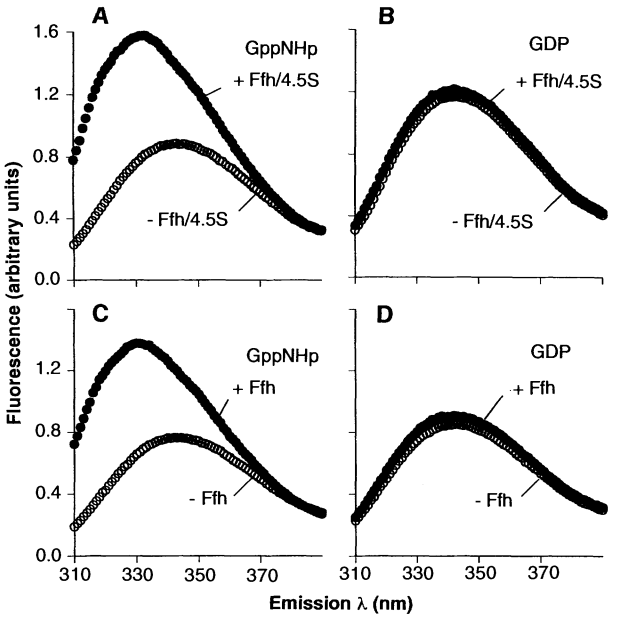

Fig. 1. Changes in tryptophan fluorescence allow monitoring of Ffh-FtsY complex formation. Fluorescence emission spectra of FtsY (1 μM) in the presence (solid circles) or absence (open circles) of 8 μM Ffh/4.5*S* RNP (**A** and **B**) or 6 μM free Ffh (**C** and **D**). Spectra in (A) and (C) were obtained under standard assay conditions (*28*) with 1 mM GppNHp-Mg^{2+} and those in (B) and (D) with 0.5 mM GDP-Mg^{2+}. Analysis of a mutant form of FtsY, FtsY(W128F), but not of FtsY(W343F), showed similar fluorescence changes upon complex formation with Ffh (*12, 17*); this indicates that the assay exclusively measures the environment of Trp^{343}, which is positioned near the nucleotide binding site. Spectra were acquired using a photon-counting SLM 8100 spectrofluorometer. An excitation wavelength of 290 nm was used. In (A) and (B), 4.5*S* RNA was added to the "–Ffh/4.5*S*" samples to control for inner filter effects.

association of $k_{on} = 5.6 \times 10^2\ M^{-1}\ s^{-1}$ and $9.2 \times 10^4\ M^{-1}\ s^{-1}$, respectively (Fig. 2, A and B, insets). These observed association rate constants are much smaller than those typically observed for protein-protein association, 10^6 to $10^8\ M^{-1}\ s^{-1}$ (*13*), which suggests that the association of a signal recognition particle (SRP) and its receptor requires conformational rearrangement.

The difference in association rate constants explains the apparent requirement for 4.5*S* RNA in previous GTP hydrolysis and binding studies. The Ffh-FtsY complex would not be expected to have formed over the time course and at the concentrations used in published assays (5 to 150 nM, 20 min). When Ffh-FtsY complex formation is driven by high concentrations of the interacting components, however, GTP hydrolysis is stimulated (*14, 15*).

The enhanced rate of complex formation might be readily explained if 4.5*S* RNA increases the affinity of FtsY for Ffh. This could arise, for example, if 4.5*S* RNA binding to Ffh preorders the protein for interaction with FtsY (*16*) or if 4.5*S* RNA interacts directly with FtsY. To test this prediction, we followed the dissociation of the Ffh/4.5*S*-FtsY and Ffh-FtsY complexes. Ffh-FtsY and Ffh/4.5*S*-FtsY complexes were preformed in the presence of GppNHp, and the change in tryptophan fluorescence was monitored as a function of time after addition of an excess of GDP (Fig. 2, C and D). After dissociation, Ffh and FtsY rapidly exchange GppNHp for GDP (*17–19*) and hence are trapped in the dissociated state.

To our surprise, the Ffh/4.5*S*-FtsY complex dissociated much faster than did the Ffh-FtsY complex (Fig. 2, C and D). The rate constant determined for the dissociation of the Ffh/4.5*S*-FtsY complex ($k_{off} = 3.3 \times 10^{-3}\ s^{-1}$) was greater than that for the Ffh-FtsY complex ($k_{off} = 1.2 \times 10^{-5}\ s^{-1}$) by a factor of 275. To confirm that the change in fluorescence observed upon addition of GDP indeed measured complex dissociation, we used two additional approaches: (i) dilution and (ii) addition of an excess of a nonfluorescent mutant FtsY [FtsY(W128F,W343F), where W = Trp and F = Phe]. Both approaches gave dissociation rate constants indistinguishable from those described above (for the Ffh/4.5*S*-FtsY complex, $k_{off} = 5.1 \times 10^{-3}\ s^{-1}$ for the first approach and $k_{off} = 6.3 \times 10^{-3}\ s^{-1}$ for the second approach)

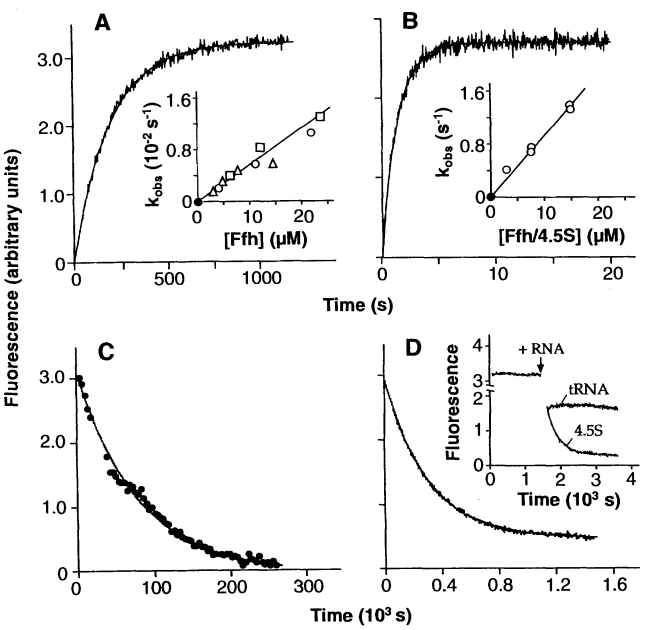

Fig. 2. Association and dissociation kinetics for the Ffh-FtsY and Ffh/4.5*S* RNP–FtsY complexes. FtsY (0.35 μM) fluorescence was monitored over time in the presence of 500 μM GppNHp-Mg^{2+} and either 7.3 μM Ffh (**A**) or 7.7 μM Ffh/4.5*S* (**B**). The data were fit to a single exponential, yielding $k_{obs} = 0.0061\ s^{-1}$ and $0.70\ s^{-1}$, respectively. Insets: Values of k_{obs} from experiments as in (A) and (B) were plotted against concentrations of Ffh and Ffh/4.5*S* RNP; different open symbols represent independent experiments in the inset of (A) [the solid circles are the experimentally determined k_{off} values from (C) and (D)]. A fit of the data to the equation $k_{obs} = k_{on}[\text{protein}] + k_{off}$ gave values of $k_{on} = 5.6\ (\pm 0.6) \times 10^2\ M^{-1}\ s^{-1}$ and $9.2\ (\pm 0.7) \times 10^4\ M^{-1}\ s^{-1}$ for the binding of Ffh and the Ffh/4.5*S* RNP, respectively, to FtsY. [The latter value is in reasonable agreement with that measured by Jagath *et al.* (*12*), $1.8 \times 10^5\ M^{-1}\ s^{-1}$, using a similar assay with GTP in place of the nonhydrolyzable GTP analog used here.] Ffh, Ffh/4.5*S* RNP, and FtsY were preincubated individually with 500 μM GppNHp-Mg^{2+} for 20 min before each initiation of the reactions. For binding reactions of Ffh/4.5*S* RNP to FtsY, measurements were made using a KinTek Stopped-Flow apparatus. (**C** and **D**) Dissociation rates of Ffh-FtsY and Ffh/4.5*S* RNP–FtsY complexes. Fluorescence intensity was monitored at 340 nm after mixing 1.5 μM Ffh-FtsY complex (C) and 2 μM Ffh/4.5*S* RNP–FtsY complex (D) with 5 mM GDP-Mg^{2+} to trap dissociated components. Fits of the data to single exponentials gave values of $k_{off} = 3.30\ (\pm 0.02) \times 10^{-3}\ s^{-1}$ and $1.20\ (\pm 0.04) \times 10^{-5}\ s^{-1}$ for dissociation of the Ffh/4.5*S* RNP–FtsY and Ffh-FtsY complexes, respectively. Reactions were carried out in triplicate. Complexes were preformed at 2× concentration in the presence of 100 μM GppNHp-Mg^{2+}. To initiate reactions, samples were diluted 1:1 in buffer containing 10 mM GDP-Mg^{2+}. Inset of (D): Dissociation of Ffh-FtsY complex was accelerated upon addition of 4.5*S* RNA. Fluorescence changes were monitored after mixing 2.5 μM Ffh-FtsY complex with 5 mM GDP-Mg^{2+}. At the time indicated (arrow), either 5.5 μM 4.5*S* RNA (lower trace) or 12 μM yeast tRNA (upper trace) was added to the sample. Fluorescence intensity changes were corrected for photobleaching (<5% of total fluorescence). The data obtained after 4.5*S* RNA addition were fit to a single exponential, yielding a rate constant of 2.7 (± 0.2) $\times\ 10^{-3}\ s^{-1}$. We also analyzed the hydrodynamic properties of the Ffh-FtsY complex by gel filtration and velocity centrifugation (*17*). In both cases, the Ffh-FtsY complex behaved as a uniform species fractionating in the size range of 100 to 150 kD, consistent with a composition of one molecule each of Ffh and FtsY.

(*17*). These rates are much slower than those measured for GppNHp release from the individual components; GppNHp release is therefore significantly slowed in Ffh-FtsY complexes, akin to "classical" GTPases that hold on tightly to bound nucleotides (*20, 21*). The decreased rate of nucleotide release from the complexes could be due to conformational changes in the nucleotide binding sites or to steric occlusion of the nucleotide exit routes.

The above results show that 4.5*S* RNA enhances dissociation of the complex between Ffh and FtsY. A prediction arising from these observations is that addition of 4.5*S* RNA to preformed Ffh-FtsY complex would facilitate its dissociation. Addition of 4.5*S* RNA does indeed increase the dissociation rate (Fig. 2D, inset). The observed factor of 200 increase is the same, within error, as that described above, indicating that the Ffh-FtsY complex is rapidly and completely converted to the faster dissociating Ffh/4.5*S*-FtsY complex. This effect was specific for 4.5*S* RNA; the addition of an equivalent amount of tRNA did not result in enhanced dissociation. These experiments show that the Ffh-FtsY complex is not irreversibly trapped in a slowly dissociating state.

The equilibrium dissociation constants calculated from the observed association and dissociation rate constants give similar values of $K_d = (k_{off}/k_{on}) = 0.024$ and 0.036 μM for the Ffh-FtsY and Ffh/4.5*S*-FtsY complexes, respectively. Equilibrium binding assays carried out with Ffh and FtsY or with Ffh/4.5*S* RNP and FtsY revealed strong binding in both cases (Fig. 3). Because of the strong binding, only upper limits could be obtained for the dissociation constants, with values of ≤0.09 μM and ≤0.017 μM for the Ffh-FtsY and Ffh/4.5*S*-FtsY complexes, respectively. These limits are consistent (within error) with the dissociation constants calculated from the kinetic data.

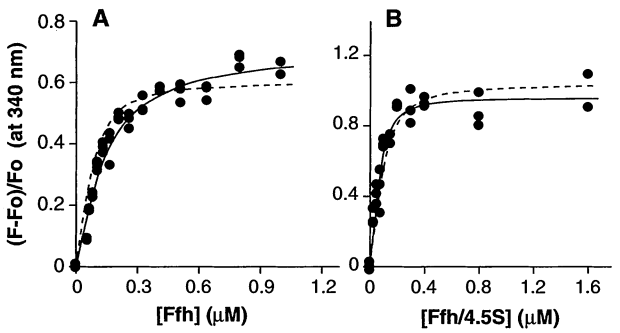

Fig. 3. Equilibrium binding of Ffh and Ffh/4.5*S* RNP to FtsY. Binding reactions were carried out for 40 hours with Ffh (**A**) and for 20 min with Ffh/4.5*S* RNP (**B**) in the presence of 0.1 μM FtsY and 500 μM GppNHp-Mg^{2+}. Because the maximal fluorescence change is observed at concentrations of Ffh and Ffh/4.5*S* near the FtsY concentration, the data were fit using a quadratic equation [solid lines; see (*25*)]. The dissociation constants obtained—0.092 (±0.038) μM and 0.016 (±0.010) μM for Ffh and Ffh/4.5*S* RNP, respectively—should be considered as upper limits for the true K_d values (*29*). The dashed lines show the binding curves calculated from the K_d values obtained from the k_{on} and k_{off} measurements in Figs. 2 and 3. Deviations in (A) are likely due to incomplete equilibration at the lower protein concentrations. Background intensities for each Ffh concentration were simultaneously measured and subtracted from each corresponding reaction before calculating the fractional fluorescence intensity change, $(F - F_o)/F_o$. For the binding reactions in (B), 4.5*S* RNA was maintained at a constant concentration of 3.0 μM in each sample to control for inner filter effects.

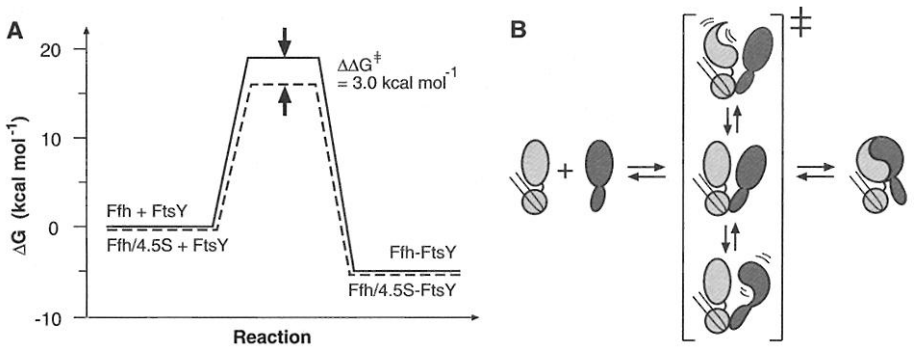

Fig. 4. Effect of 4.5*S* RNA on the Ffh-FtsY binding reaction. (**A**) Free energy–reaction profile for Ffh-FtsY association in the absence (solid line) and presence (dashed line) of bound 4.5*S* RNA. The relative energy levels are shown for a standard state of 1 M and were calculated from the observed association and dissociation rate constants using the equation $\Delta G = -RT \ln(kh/k_BT)$ (*13*), where $R = 1.987$ kcal mol^{-1} K^{-1}, Boltzmann's constant $k_B = 3.3 \times 10^{-27}$ kcal K^{-1}, $h = 1.58 \times 10^{-37}$ s^{-1}, and $T = 298$ K. (**B**) Schematic diagram depicting a model for how 4.5*S* RNA may act catalytically in Ffh-FtsY complex formation. In this model, 4.5*S* RNA (hairpin) helps form a transient tether between Ffh (light gray) and FtsY (dark gray), which allows a complex to form long enough for Ffh, FtsY, or both (as depicted) to obtain the correct conformation to become more stably locked. The presence of the RNA lowers the energy barrier to this "transition state(s)" within the brackets by ~3 kcal mol^{-1}, as indicated by the arrows in (A). The tether would be transient, however, as the complex is not measurably stabilized in the presence of the RNA.

Taken together, the above data show that 4.5*S* RNA not only speeds formation of the Ffh-FtsY complex by a factor of ~200, but also accelerates its dissociation to a similar extent. In analogy to an enzymatic reaction, the RNA therefore stabilizes a transition state for the binding reaction, lowering the energetic barrier separating free and complexed components by ~3 kcal mol^{-1} (Fig. 4A). 4.5*S* RNA therefore carries out a "catalytic" function in the assembly reaction. In contrast to conventional catalysts that facilitate multiple reactions when present in substoichiometric amounts, 4.5*S* RNA remains tightly bound to Ffh in a stoichiometric complex.

How could the 4.5*S* RNA stabilize the transition state for association and dissociation without substantially altering the equilibrium for protein-protein association (Fig. 4A), and what might be the meaning of these observations for SRP function? A plausible model to account for the catalytic behavior is that 4.5*S* RNA can serve as a transient tether, linking the two interacting proteins temporarily. Transient tethering would lengthen the time window after the initial collisional encounter of the components during which they can convert to the stably bound complex (Fig. 4B). Within a transiently formed complex, the two proteins might be able to find the rare conformations within or between the proteins that are competent for stable binding. As noted above, the observation that the association rate constant, even in the presence of RNA, is much lower than typically observed for protein-protein interactions suggests a requirement for such rearrangements before formation of the stable complex.

The simplest molecular model posits a direct role of the 4.5*S* RNA in providing the transient tether. Alternatively, the RNA could form the tether together with part of the Ffh protein or could induce conformational changes in Ffh in a region, such as the M domain of Ffh to which 4.5*S* RNA binds (*16, 22, 23*), that then serves as the tether. In either case, the stable complex might result from direct interaction of structurally related GTPase domains (NG domains) of Ffh and FtsY, as is suggested from the reciprocal stimulation of GTP hydrolysis (*7*). Furthermore, mutagenesis studies show that the tryptophan responsible for observed fluorescence changes resides in the NG domain of FtsY (Fig. 1) (*12*). It is critical to emphasize that, according to the transient tether model, the region used as the tether would not be involved in stabilizing contacts in the final complex. Consistent with this notion, no changes in the footprint of Ffh on 4.5*S* RNA were observed upon binding to FtsY (*17*).

Although we have characterized here the

consequences of the presence or absence of 4.5*S* RNA for Ffh-FtsY complex formation, an extension of these results suggests that the catalytic activity of 4.5*S* RNA could serve as a built-in regulator for the SRP targeting cycle. As the M domain also contains the signal sequence binding site of Ffh (*22*), signal sequence binding could induce minor changes in RNA conformation (on the order of 3 kcal mol^{-1}, i.e., breaking or forming only a few hydrogen bonds), which in turn could lead to changes in kinetics controlling Ffh-FtsY complex formation. Indeed, the structure of SRP RNA bound to the Ffh M domain shows that SRP RNA is closely juxtaposed to the signal sequence binding pocket, which underscores the feasibility of direct crosstalk between bound signal sequences and the RNA (*24, 25*). It is therefore an appealing hypothesis that protein targeting by a SRP and its receptor could be controlled through conformational changes in the RNA in addition to nucleotide occupancy of the GTPase domains. According to this view, SRP RNA would play a much more active role than previously presumed in regulating the interaction between the two GTPases.

The catalytic properties shown here for 4.5*S* RNA may not be unique to this system but may be a paradigm for other ribonucleoprotein assemblies, such as spliceosomes and ribosomes, that go through a dynamic cycle (*26, 27*). Like SRP-mediated protein targeting, these biological processes depend on the coordinated formation and dissociation of complex ribonucleoprotein assemblies. Perhaps the RNA components in these complexes affect conformational changes in a manner analogous to 4.5*S* RNA and thus provide new means for regulation by modulating the kinetic parameters that govern complex formation and disassembly.

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28. Fluorescence spectra were obtained and reactions carried out in 50 mM Hepes (pH 7.5), 150 mM KOAc, 1.5 mM $Mg(OAc)_2$, 0.01% (v/v) Nikkol detergent, and 2 mM dithiothreitol at 25°C (Ac, acetyl). *Escherichia coli* Ffh, 4.5S RNA, and FtsY were prepared as described (*5*). Ffh/4.5S RNP was assembled using a twofold molar excess of RNA over Ffh and used without further purification (*6*).

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Uninterrupted MCM2-7 Function Required for DNA Replication Fork Progression

Karim Labib, José Antonio Tercero, John F. X. Diffley*

Little is known about the DNA helicases required for the elongation phase of eukaryotic chromosome replication. Minichromosome maintenance (MCM) protein complexes have DNA helicase activity but have only been functionally implicated in initiating DNA replication. Using an improved method for constructing conditional degron mutants, we show that depletion of MCMs after initiation irreversibly blocks the progression of replication forks in *Saccharomyces cerevisiae*. Like the *Escherichia coli* dnaB and SV40 T antigen helicases, therefore, the MCM complex is loaded at origins before initiation and is essential for elongation. Restricting MCM loading to the G_1 phase ensures that initiation and elongation occur just once per cell cycle.

The six MCM proteins of the MCM2-7 family are assembled at the end of mitosis into prereplicative complexes (pre-RCs) at origins of DNA replication and are essential for origin activation to occur as cells enter the S phase [reviewed in (*1*, *2*)]. A subcomplex of three human MCM proteins, MCM4, -6, and -7, has limited DNA helicase activity in vitro (*3*, *4*), and an archaeal protein related to the MCM2-7 family also functions as a DNA helicase (*5*, *6*), suggesting that MCM proteins may be required for strand unwinding during the initiation step of chromosome replication. After initiation, MCM proteins become redistributed away from origins (*7*), but previous genetic analysis indicated that MCM proteins are not required for chromosome replication to be completed after origin activation (*8*, *9*), which supports arguments against an essential role for MCM proteins in the progression of replication forks.

To clarify the in vivo role of MCM proteins during chromosome replication, we generated a novel set of conditional *mcm* mutants. Initially, we used the "heat-inducible degron" method of Dohmen, Wu, and Varshavsky (*10*), whereby the target gene is altered such that an NH_2-terminal extension (the degron) is added to the protein of interest, specifically reducing the half-life of the fused product at 37°C. However, we found that degradation of such fusion proteins at 37°C is inefficient and inactivation of protein function is incomplete. For example, when a *mcm7-td* mutant is shifted to 37°C, the level of Mcm7-td protein declines slowly and can still be detected after 2 hours at 37°C [Fig. 1A, (i)], and cells arrest with a DNA content between 1C (the amount of DNA in a haploid cell in the G_1 phase) and 2C (the amount of DNA in a haploid cell in the G_2 phase) [Fig. 1B, (i)]. To improve the method, we took advantage of the fact that expression of *UBR1*, the gene encoding the degron recognition factor, can be regulated by the galactose-inducible *GAL1-10* promoter without inhibiting cell growth. In the absence of *UBR1* expression, the level of Mcm7-td protein is unchanged after shifting to 37°C [Fig. 1A, (ii)] and colony formation is not inhibited (*11*). However, when cells are grown in the presence of galactose to induce high levels of *GAL-UBR1* expression, Mcm7-td protein is present at 24°C but is very rapidly de-

Imperial Cancer Research Fund Clare Hall Laboratories, South Mimms, Hertfordshire, EN6 3LD, UK.

*To whom correspondence should be addressed. E-mail: J.Diffley@icrf.icnet.uk