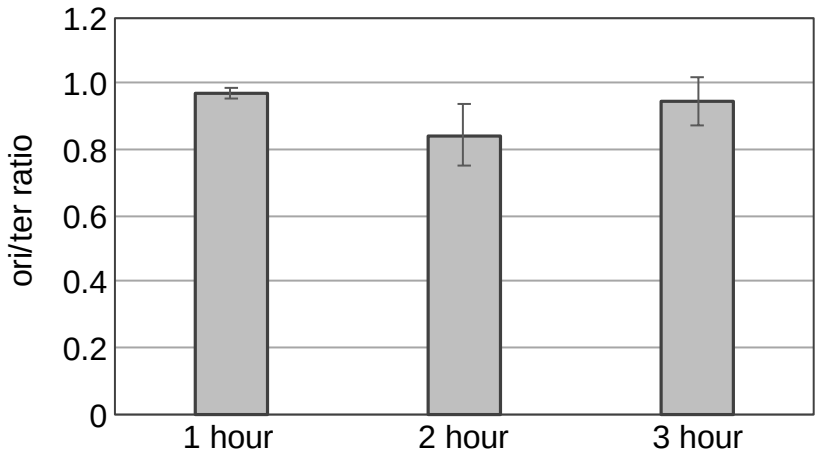
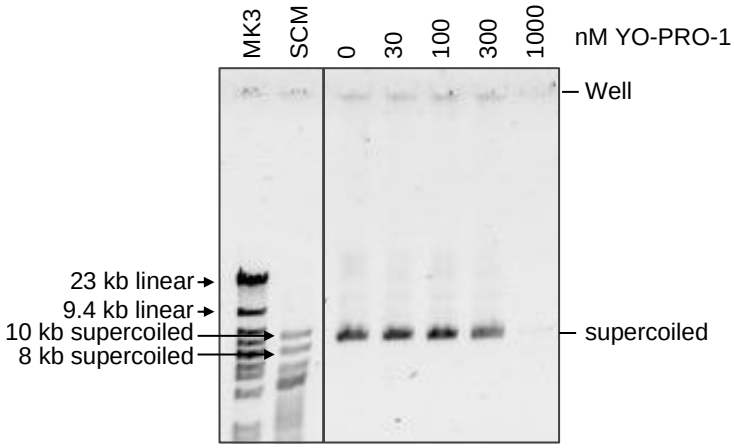
Supplementary information

Real-Time Analysis of Initiation Regulation Systems During the Progression of the Reconstituted Chromosomal Replication Cycle



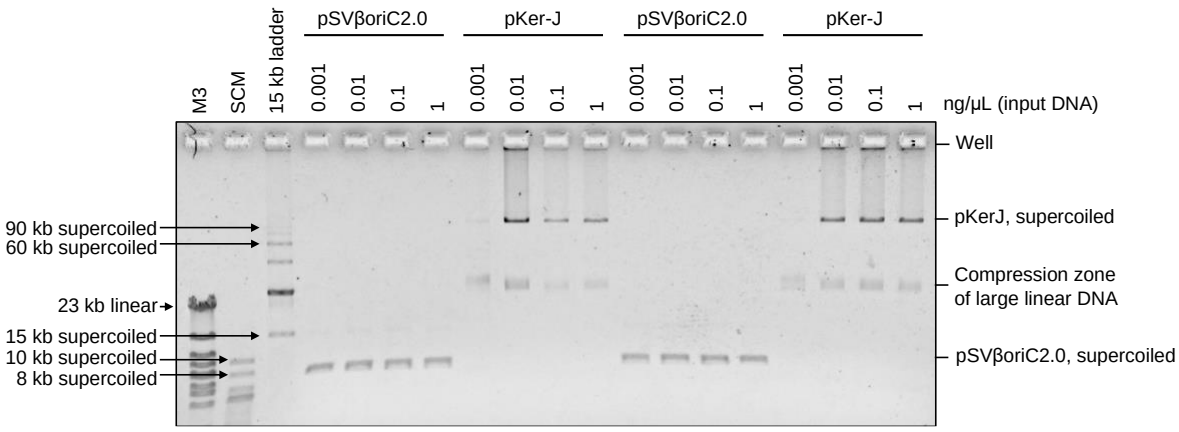
Supplementary Fig. S1. ori/ter ratio of RCR product at different time points

2 ng/μL pMSR227 (205 kb) was propagated in the RCR mixture (4 μL) for 3 hour. The RCR roduct was subjected to qPCR determination of ori/ter ratio as described in previous study[3]. The ori/ter ratio was normalized using the pMSR227 supercoiled template that was similary analyzed by qPCR. The ratio, averaged from two qPCR samples from the same RCR product at each time point, are shown with the error bar (standard error of the mean).



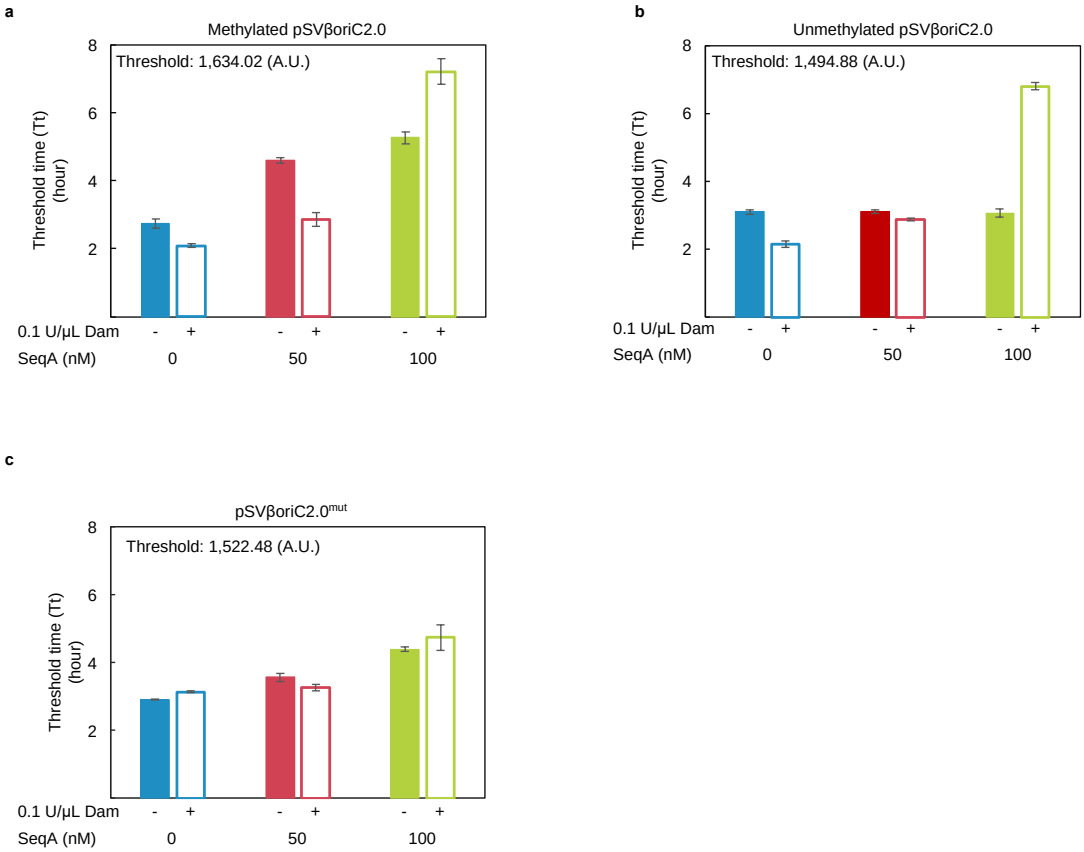
Supplementary Fig. S2. Effect of YO-PRO-1 iodide on DNA propagation in RCR

pSVβoriC2.0 (0.1 ng/μL) was incubated in RCR mixture at 30˚C for 6 h in the presence or absence of YO-PRO-1 iodide. To analyze propagated DNA products, RCR products were diluted 2:10 in 1x RCR buffer and incubated at 30˚C for 30 min to finalize replication intermediates. A 0.5 μL sample were analyzed using 0.5% TBE-agarose gel electrophoresis. The gel was stained with dsGreen, and imaging was performed using Typhoon9500. Size-marker fragments (M3) were derived from phage DNA. SCM represents a supercoiled DNA marker containing supercoiled plasmids.



Supplementary Fig. S3. Agarose gel electrophoresis analysis of the real-time RCR products

Portions (0.1 μL) of the final products from the real-time RCR experiments in Fig. 2a and 2d were analyzed using 0.5% TBE-agarose gel electrophoresis. The gel was stained with dsGreen, and imaging was performed using Fusion. Size-marker fragments (M3) were derived from phage DNA. SCM represents a supercoiled DNA marker containing supercoiled plasmids, while the 15-kb ladder includes monomeric and concatemeric 15-kb plasmids. Open circular DNA was trapped in the wells, whereas supercoiled and linear DNA migrated into the gel.



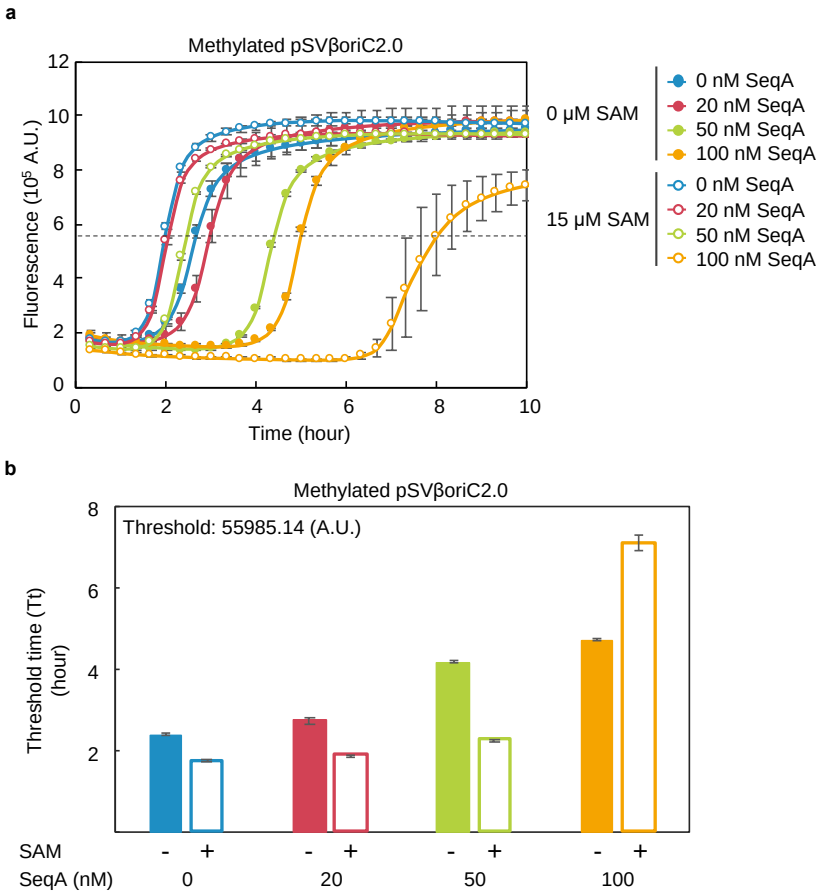
Supplementary Fig. S4. Threshold time in real-time RCR affected by SeqA and Dam methylase

(a) Threshold times (Tt) from Fig. 3b were determined as the time required for DNA propagation to cross the threshold. The average values and standard error of the mean (SEM) from two samples per experiment are reported. The threshold times were 164.9 ± 8.4 min (Δ0.1 U/μL Dam, 0 nM SeqA), 125.6 ± 3.1 min (0.1 U/μL Dam, 0 nM SeqA), 276.3 ± 4.3 min (Δ0.1 U/μL Dam, 50 nM SeqA), 171.6 ± 11.7 min (0.1 U/μL Dam, 50 nM SeqA), 316.3 ± 10.3 min (Δ0.1 U/μL Dam, 100 nM SeqA), and 433.0 ± 22.8 min (0.1 U/μL Dam, 100 nM SeqA). **(b)** Threshold times from Fig. 3c demonstrated similar variations depending on the concentration of SeqA and Dam methylase. The threshold times were 186.3 ±3.8 min (Δ0.1 U/μL Dam, 0 nM SeqA), 129.2 ±5.5 min (0.1 U/μL Dam, 0 nM SeqA), 186.9 ±2.9 min (Δ0.1 U/μL Dam, 50 nM SeqA), 173.4 ±2.6 min (0.1 U/μL Dam, 50 nM SeqA), 184.3 ±7.9 min (Δ0.1 U/μL Dam, 100 nM SeqA), and 408.6 ±6.4 min (0.1 U/μL Dam, 100 nM SeqA). **(c)** Threshold times from Fig. 3d further highlighted the impact of these regulatory elements. The threshold times were 173.7 ± 0.7 min (Δ0.1 U/μL Dam, 0 nM SeqA), 187.6 ± 1.4 min (0.1 U/μL Dam, 0 nM SeqA), 213.3 ± 6.9 min (Δ0.1 U/μL Dam, 50 nM SeqA), 195.8 ± 5.6 min (0.1 U/μL Dam, 50 nM SeqA), 263.2 ± 3.5 min (Δ0.1 U/μL Dam, 100 nM SeqA), and 284.2 ± 23.0 min (0.1 U/μL Dam, 100 nM SeqA), respectively.

Plasmid	0.1 U/μL Dam	SeqA (nM)	Doubling time (min)
Methylated pSVβoriC2.0	-	0	40.6 ± 3.47
		50	35.0 ± 0.66
		100	33.2 ± 1.30
	+	0	30.9 ± 2.81
		50	29.9 ± 0.00
		100	30.1 ± 2.23
Unmethylated pSVβoriC2.0	-	0	35.8 ± 2.17
		50	38.2 ± 2.13
		100	34.0 ± 0.24
	+	0	29.6 ± 1.12
		50	30.8 ± 1.51
		100	24.4 ± 0.71
pSVβoriC2.0^{mut}	-	0	45.0 ± 0.12
		50	42.4 ± 0.37
		100	40.0 ± 4.94
	+	0	44.7 ± 0.15
		50	45.1 ±1.51
		100	44.0 ± 3.06

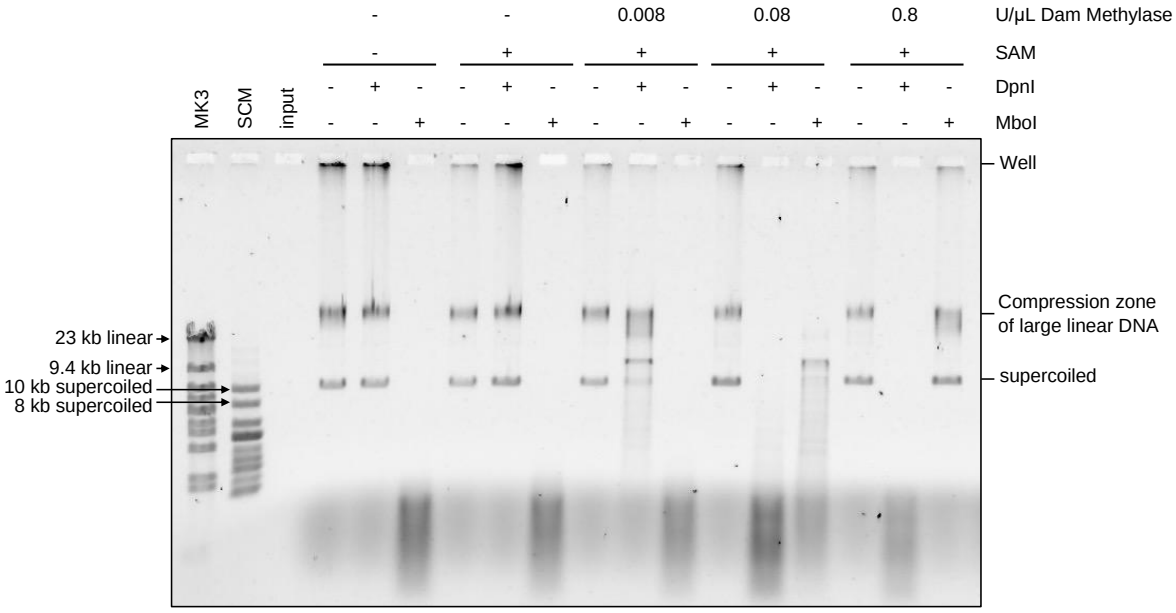
Fig. S5. Doubling time in the real-time RCR experiments in Fig. 3

Doubling times were calculated from (Fig. 3b) for Methylated pSVβoriC2.0, (Fig. 3c) for unmethylated pSVβoriC2.0, and (Fig. 3d) for pSVβoriC2.0^{mut}.



Supplementary Fig. S6. Effect of SAM on the RCR repression by SeqA and *oriC* methylation

(a) Propagation of DNA during real-time RCR. Real-time RCR was performed with 0.01 ng/μL of methylated pSVβoriC2.0 in the presence of 0.1 U/μL Dam methylase, with or without the indicated concentrations of SeqA and SAM, as described in Fig. 2a except that RecG, RecJ, and *ExoIII* were excluded. Data represent the average of two samples from the same experiment, with error bars showing the standard error of the mean. Dotted horizontal lines represent thresholds, calculated as the median value between the maximum and minimum fluorescence based on the results in the absence of both SAM and SeqA: 55985.14. **(b)** Threshold times (Tt) from Supplementary Fig. S6a were determined as the time required for DNA propagation to cross the threshold. The average values and standard error of the mean (SEM) from two samples per experiment are reported. Tt values were 143.4 ± 1.4 min (0 μM SAM, 0 nM SeqA), 164.1 ± 4.2 min (0 μM SAM, 20 nM SeqA), 259.6 ± 0.4 min (0 μM SAM, 50 nM SeqA), and 283.1 ± 0.4 min (0 μM SAM, 100 nM SeqA) , 104.9 ± 0.4 min (15 μM SAM, 0 nM SeqA), and 113.1 ± 1.1 min (15 μM SAM, 20 nM SeqA) , 136.3 ± 0.9 min (15 μM SAM, 50 nM SeqA), and 425.9 ± 11.1 min (15 μM SAM, 100 nM SeqA).



Supplementary Fig. S7. Restriction enzyme analysis of methylation state of RCR propagated DNA in the presence of Dam methylase and SAM

oriC-containing circular DNA (pOri8, 9.5 kb, 0.1 ng/μL) was incubated in RCR at 30˚C for 3 hours in the presence or absence of 80 μM SAM and/or indicated concentration of Dam methylase. Aliquotes (0.5 μL) were then digested by DpnI or MboI at 30˚C for 30 min. Sample without a restriction enzyme were also analyzed as negative controls. Portions equivalent to 0.1 μL RCR products were analyzed using 0.5% TBE-agarose gel electrophoresis. The gel was stained with dsGreen, and imaging was performed using Typhoon9500. Size-marker fragments (M3) were derived from phage DNA. SCM represents a supercoiled DNA marker containing supercoiled plasmids.

Supplementary Tables

Supplementary Table S1. The *oriC* cassette

Name	Sequence (5' -> 3')	
oriC cassette	TGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAA GAGAGCTGTTGACAGAGGGTCATtttcacactataatgcagtgaatcccaaacagtatgttgtacctaaaggatcctgg gtattaaaaagaagatctatttatttagagatctgttctattgtgatctcttattaggatcgcactgccctgtggataacaaggatccggcttttaaga tcaacaacctggaaaggatcattaactgtgaatgatcggtgatcctggaccgtataagctgggatcagaatgaggggttatacacaactcaaa aactgaacaacagttgttctttggataactaccggttgatccaagcttcctgacagagttatccacagtagatcgcacgatctgtcagctcatttc ctttaggtacaacatactagaatatttgcctacagcctcctttcggaggctgttttttttATCAAAGGATCTTCTTGAGATCCTT TTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAA	Lower-case letters indicate the wild type *oriC* sequence (264 bp)

Minimal *oriC* is underlined. The *ter* sequences are surrounded by a square. Capital letters indicate the overlapping ends.

Supplementary Table S2. List of primers

Name	Sequence (5' -> 3')	
SUE10623	CTTCTTTTTAATACCCAGGAACCCtttaggtacaacatactgtttgggattcactgcattatagtgtgaa aatgaccctctgtcaacagc	Reverse for GTTC *oriC*
SUE10624	GGGTTCCTGGGTATTAAAAAGAAGttctatttatttagagttctgttctattgtgttctcttattaggttcg caCTGCCCTGTGGATAACAAGG	Forward for GTTC *oriC*
SUE10626	GTGTTCCTGGACCGTATAAGCtgggttcagaatgaggggttatacacaactcaaaaactgaacaaca GTTGTTCTTTGGATAACTACCGG	Forward for GTTC *oriC*
SUE10627	GGAAATGAGCTGACAGATCGTGCgaactactgtggataactctgtcaggaagcttggaacaaCC GGTAGTTATCCAAAGAACAAC	Reverse for GTTC *oriC*
SUE10628	GCACGATCTGTCAGCTCATTTCCtttaggtacaacatactagaatatttgcctacagcctcctttcgga ggctgtttttttatc	Forward for GTTC *oriC*
SUE10713	GCTTATACGGTCCAGGAACACcgaacattcacagttaatgaacctttccaggttgttgaacttaaaagc cggaaCCTTGTTATCCACAGGGCAG	Reverse for GTTC *oriC*

Capital letters indicate the overlapping ends.