Spermidine strongly increases the fidelity of *Escherichia coli* CRISPR Cas1–Cas2 integrase

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Received for publication, January 18, 2019, and in revised form, June 3, 2019. Published, Papers in Press, June 6, 2019. DOI 10.1074/jbc.RA119.007619

Edited by Patrick Sung

Site-selective CRISPR array expansion at the origin of bacterial adaptive immunity relies on recognition of sequence-dependent DNA structures by the conserved Cas1–Cas2 integrase. Off-target integration of a new spacer sequence outside canonical CRISPR arrays has been described in vitro. However, this nonspecific integration activity is rare in vivo. Here, we designed gel assays to monitor fluorescently labeled protospacer insertion in a supercoiled 3-kb plasmid harboring a minimal CRISPR locus derived from the *Escherichia coli* type I-E system. This assay enabled us to distinguish and quantify target and off-target insertion events catalyzed by *E. coli* Cas1–Cas2 integrase. We show that addition of the ubiquitous polyamine spermidine or of another polyamine, spermine, significantly alters the ratio between target and off-target insertions. Notably, addition of 2 mM spermidine quenched the off-target spacer insertion rate by a factor of 20-fold, and, in the presence of integration host factor, spermidine also increased insertion at the CRISPR locus 1.5-fold. The observation made in our in vitro system that spermidine strongly decreases nonspecific activity of Cas1–Cas2 integrase outside the leader-proximal region of a CRISPR array suggests that this polyamine plays a potential role in the fidelity of the spacer integration also in vivo.

In the prokaryotic adaptive immune response, foreign DNA is processed to produce DNA pieces that are at the origin of the insertion of new spacers in a CRISPR array (1–6). The insertion occurs at the leader-repeat boundary of the array (1, 7–10). However, insertion at off-target sites was also observed. Occurrence of these events is rather frequent in vitro but rare in vivo (11–13).

Protospacer insertion at the CRISPR locus has been the object of numerous studies (14–24). A complex of Cas1 and Cas2 proteins, consisting of two Cas1 dimers bridged by a central Cas2 dimer, guides the insertion (25–28). To obtain integration, protospacers must be bound by Cas1–Cas2 as a dual-forked DNA (4, 17, 26, 29). Nucleophilic attack of the terminal 3'-OH group at the end of one 3'-overhang covalently connects the protospacer to one strand of the target DNA. The opposite 3'-overhang triggers the second step of integration through covalent binding of its 3'-OH group with the other strand of target DNA (13, 26, 29). The two-step reaction needs the presence of magnesium (13). In vitro studies suggest that the first step preferentially occurs at the leader-repeat boundary of the CRISPR locus (12, 28, 30, 31). However, primary integration at the opposite boundary of the first repeat in the array could also be observed if supercoiled plasmid was the recipient DNA (12, 13).

Spacer acquisition at the leader-repeat border in the *Escherichia coli* Type I-E CRISPR system depends on the integration host factor (IHF) (12, 31), a heterodimer that binds DNA at specific sites (32). Recent cryo-EM structure of the *E. coli* CRISPR integration complex including IHF showed that this accessory protein sharply bends target DNA, bringing an upstream recognition motif (31) into contact with one of the noncatalytic Cas1 subunits (27). Both this motif and the IHF-binding site lie within the 60-bp leader segment adjoining the first CRISPR repeat and are necessary for spacer acquisition in vivo (10, 12, 31).

IHF is well-known to induce sharp bends in the helical axis of dsDNA (33). Moreover, IHF-induced DNA bends are documented to contribute to chromosome organization, site-specific recombination, DNA replication, and gene expression in various bacteria (32, 34–36). Interference of polyamines in such biological processes has been frequently reported (37–41). These studies prompted us to search for polyamine influence on the action of Cas1–Cas2 in spacer acquisition. For this purpose, we followed integration of a model protospacer in a supercoiled CRISPR-containing plasmid. After plasmid digestion and agarose gel electrophoresis, specific and off-target integration events could be distinguished and quantitated. We found that, in the presence of millimolar spermidine concentrations, off-target integration reaction rates were strongly repressed, whereas the specific one was slightly increased. The improvement of the fidelity of the integration reaction at the CRISPR locus required the presence of both IHF and a native IHF-binding site.

Results

Products of the target and off-target integration reactions can be distinguished and quantitated through electrophoresis experiments

In a previous study (42), we developed an electrophoretic mobility shift assay using a 5' fluorescently labeled model pro-
tospacer and a supercoiled CRISPR-containing plasmid as the acceptor to demonstrate that DNA-binding specificities of \( E. \ coli \) Cas1–Cas2 integrase drive its recruitment at a CRISPR locus. Here, we used agarose gel electrophoresis to follow DNA products resulting from the first and second steps of protospacer integration. Accumulation of such products was triggered by addition of 10 \( \text{mM} \) magnesium ions to a mixture containing 5 \( \text{mM} \) EDTA, 7.5 \( \text{nm} \) of a supercoiled 3-kbp plasmid DNA (pF0) carrying a minimal CRISPR locus limited to 62 bases of the leader region plus one 28-bp repeat, 7.5 \( \text{nm} \) Cas1–Cas2 integrase, 7.5 \( \text{nm} \) of a 33-bp protospacer twice 5\(^{-}\)-labeled with two different fluorophores (\text{red} and \text{green} in the figures), and IHF in excess (40 \( \text{nm} \)). After incubation at 37 °C for various times (0–60 min), the reaction was quenched by excess EDTA and SDS additions. To distinguish canonical integration at the CRISPR locus from off-target reactions, the plasmid was further cleaved into two fragments (A and B) through restriction enzyme digestion (HindIII plus KpnI) prior to agarose gel electrophoresis (see Fig. 1 and Fig. S1). Short fragment B (133 bp) carried the CRISPR locus. The much larger fragment A underwent off-target integration events.

A routine gel obtained in this way is shown in Fig. 1. Controls without magnesium (lanes 1 and 2) show the free fluorescent protospacer band only (band 1). Upon magnesium addition, three bands appeared over that of the protospacer. The heaviest (band 4) corresponds to the bigger DNA restriction fragment (A) fluorescently labeled by covalent attachment of the protospacer at off-target integration sites (12, 13). We expected that bands 2 and 3 resulted from various products of protospacer integration at the CRISPR locus on the smaller DNA restriction fragment (B). In agreement with this idea, these two bands could no longer be detected if a control plasmid devoid of the CRISPR leader-repeat region (pF1) was used (lane 13). In addition, absence of labeling of the CRISPR-free fragment B obtained from pF1 indicated that, in our experimental conditions, the small DNA region between the HindIII and KpnI sites did not offer any detectable off-target site for integration.

To more precisely assign bands 2 and 3 originating from plasmid pF0, time of incubation with magnesium was varied. At the shortest times, upper band 3 was the most visible (Fig. 1, lanes 4 and 5). At the longest times, the faster band 2 increased in intensity (lanes 6–8). This result raised the possibility that bands 3 and 2 corresponded to half- and full-site integrations, respectively. To confirm this assignment, a preparative agarose gel was performed after 1-h incubation (see the legend to Fig. 2). DNA bands from bands 2 and 3 were extracted and submitted to PAGE under denaturing conditions. Electrophoresis of agarose-band 3 revealed three bands (Fig. 2B, lane 2). The middle band size (~99 bases) fitted with reaction of the protospacer at the leader-repeat junction, the biggest size (~128 bases) agreed with reaction at the other extremity of the repeat (Fig. 2A), and the smallest one corresponded to the size of each strand of the protospacer (33 bases). All three bands were more or less greenish-yellow in Fig. 2B, indicating that either extremity of our model protospacer reacted with acceptor DNA. Upon agarose-band 2 analysis (Fig. 2B, lane 1), only the two upper bands (~99 and ~128 bases) were observed (yellow). The 33-base oligonucleotides composing the protospacer were absent. This strongly indicated that agarose-band 2 was a full-integration product of the reaction with the acceptor plasmid.

We also performed an agarose gel experiment with the same protospacer but missing the 3′-OH group at one of its 3′-end (protospacer P1–3′H, see Fig. 1). This protospacer was 5′-DY682-labeled (red) at its right end. Band 2 was lacking on the agarose gel (Fig. 1, lanes 10 and 11), in agreement with its assignment above as a full-site integration product.

Another experiment was undertaken with model protospacer (P1) and an acceptor DNA in which the whole 28-bp repeat sequence was lacking (pF5, Fig. S2). Such a plasmid is, in...
principle, unable to achieve the second step of the integration reaction (27). Band 3 was formed (Fig. 1, lanes 15–17), not band 2, a behavior showing again that band 2 corresponded to a full-site integration product.

Finally, we directly submitted our restricted DNA samples to denaturing PAGE. With the intact protospacer, bands of ~99 and ~128 bases were again observed (Fig. 2C, lanes 2–6), even at the shortest incubation time (0.5 min). Yield of integration at the leader-repeat junction (band at ~99 bases) was favored ~2-fold over that at the opposite end of the repeat (band at ~128 bases). The bands exhibited ~1.6-fold more green than red fluorescence, in agreement with previous results showing that a C at the 3′-end of one protospacer strand favors integration (13). With the protospacer lacking one of its 3′-OH ends (P1–3′H), there were the same two bands (Fig. 2C, lanes 9 and 10). Explanation of such results needs to assume that the first integration event may occur at either end of the CRISPR repeat (see Fig. 2A). The gel of Fig. 2C (lanes 5 and 6, incubation times of 6 and 10 min, respectively) also showed heavy bands (>280 bases) that could only be accounted for by non-CRISPR-dependent integration reactions in the larger DNA restriction product (fragment A, see Fig. 1). A control experiment with pF1, which lacks the canonical CRISPR locus, displayed the same heavy bands (Fig. 2C, lane 11, incubation time 10 min). In line with this observation, submission to PAGE of isolated band 4, as produced on a preparative agarose gel from restricted pF0, also showed the heavy bands (Fig. 2C, lane 3). An additional experiment with direct application of the restricted reaction sample on the polyacrylamide gel was carried out with pF1 incubated for 60 min with a 4-fold higher concentration of both Cas1–Cas2 integrase and spermidine. The intensity of these contaminants represented less than 1% of the total fluorescence of the samples. Some aggregation of DNA products occurred during sample processing, as evidenced by the detection of a minor fluorescent signal in the wells. Size markers were made as described under “Experimental procedures.”

Millimolar concentration of spermidine markedly reduces the appearance rate of off-target integration products

DNA products resulting from protospacer integration were followed as a function of the concentration of spermidine, a ubiquitous natural polyamine that bears three amino groups.
Cas1–Cas2 integrase and spermidine

The reaction mixture consisted of supercoiled plasmid carrying the CRISPR locus (pF0), Cas1–Cas2 integrase, double 5′-fluorolabeled model protospacer (P1), IHF, and various concentrations of the polyamine. Reaction was initiated with magnesium and quenched with EDTA and SDS after 2 min. We verified that spermidine could not substitute for magnesium in sustaining the integration reaction. As shown in Fig. S3, covalent reaction of the protospacer at or outside the CRISPR locus increased almost linearly with time during the first 2 min of incubation (gray and blue curves in panel A). Therefore, taking a 2-min time-window ensured measurements of initial rate values of the integration reactions.

The result of a typical agarose gel after plasmid DNA restriction is shown in Fig. 3. As deduced from the sum of fluorescence intensities of bands 2 and 3, the rate of integration at the CRISPR locus smoothly increased with spermidine concentration, reaching a maximum (~1.5-fold) at 5 mM of the polyamine (Fig. 4A). Beyond this concentration, severe inhibition of integration occurred. Such a drop in activity upon addition of polyamines has already been reported in the cases of several systems involving DNA as the substrate (43–45). This phenomenon is generally believed to reflect aggregation of DNA (46). In our hands, plasmid DNA precipitation is likely to account for such a quenching of all specific integration reactions (Fig. S4). Outside the CRISPR target region, integration (band 4) decreased to near zero as soon as spermidine addition exceeded 1 mM. However, at 0.1–0.2 mM spermidine, stimulation (2–3–fold) of band 4 intensity could be reproducibly observed (Figs. 4A and 5).

We defined a specificity index as the ratio between the initial rates of the integration events at the CRISPR locus (band 2 + band 3 intensities) and outside (band 4 intensity). This index varied from ~10 in the absence of spermidine to more than 300 in the presence of 2–5 mM of this polyamine (Fig. 4A).

Experiments were also performed with l-lysine or with polyamines bearing either four (spermine) or two (putrescine, cadaverine) amino groups. Spermine behaved very similarly to spermidine, being, however, effective at a 10-fold lower concentration than spermidine (Fig. 4B). With putrescine, cadaverine, and l-lysine, some improvement of the specificity index could be also detected, although much reduced as compared with that observed with spermidine or spermine (Fig. 4, C–E). Neither addition of CaCl2, which is known to favor DNA condensation (47), nor of excess KCl or MgCl2 reproduced the effects of spermidine or spermine (Fig. 4, F–H).

Because spermine and spermidine co-exist in some organisms, we wondered whether a combination of these two polyamines had a special effect on Cas1–Cas2-catalyzed protospacer integration. Concomitant presence of spermidine and spermine produced results consistent with the addition of the potencies of each polyamine, thereby indicating additivity of the effects of spermidine and spermine, not synergy or antagonism (Fig. S5).

To determine whether spermidine had the same effects on the first and second integration steps at the CRISPR locus, we followed intensities of bands 2 and 3 as a function of time (0–60 min), in the absence or presence of 2 mM spermidine. The gels and the deduced kinetics in Fig. S3 showed that the kinetics of band 2 and band 3 intensities were similarly up-shifted by the presence of spermidine, thus precluding a major effect of the polyamine on the transition from half- to full-site integration. In contrast, comparison of the two band 4 kinetics confirms the inhibition of the off-target reactions by the polyamine.

To know more on the quenching of band 4 intensity by spermidine, we used the non-CRISPR plasmid pF1 instead of pF0. As in the case of the pF0-band 4, an increase in the spermidine concentration reduced the intensity of pF1-band 4, although the inhibition was less pronounced (Fig. 5A). We imagined that...

Figure 3. Effect of spermidine on protospacer integration into CRISPR plasmid pF0. Reaction mixtures, incubated for 2 min, contained 7.5 nM protospacer P1 (Fig. 1), 7.5 nM Cas1–Cas2, 40 nM IHF, 7.5 nM supercoiled plasmid pF0, and the indicated concentrations of spermidine. Samples were analyzed by native agarose gel electrophoresis as explained in the legend to Fig. 1. The gel was scanned for fluorescence of DY682 (red) and DY782 (green) and the resulting images were superimposed. Shown are parts of the gel corresponding to unreacted protospacer (band 1), integration at the CRISPR locus smoothly increased with spermidine concentration, reaching a maximum (~1.5-fold) at 5 mM of the polyamine (Fig. 4A). Beyond this concentration, severe inhibition of integration occurred. Such a drop in activity upon addition of polyamines has already been reported in the cases of several systems involving DNA as the substrate (43–45). This phenomenon is generally believed to reflect aggregation of DNA (46). In our hands, plasmid DNA precipitation is likely to account for such a quenching of all specific integration reactions (Fig. S4). Outside the CRISPR target region, integration (band 4) decreased to near zero as soon as spermidine addition exceeded 1 mM. However, at 0.1–0.2 mM spermidine, stimulation (2–3–fold) of band 4 intensity could be reproducibly observed (Figs. 4A and 5).

We defined a specificity index as the ratio between the initial rates of the integration events at the CRISPR locus (band 2 + band 3 intensities) and outside (band 4 intensity). This index varied from ~10 in the absence of spermidine to more than 300 in the presence of 2–5 mM of this polyamine (Fig. 4A).
this discrepancy resulted from a recruitment of Cas1–Cas2 at the CRISPR locus of pF0 in the presence of spermidine, thereby limiting the Cas1–Cas2 free concentration available for off-target reactions. An additional experiment was thus made at a 10-fold higher concentration of Cas1–Cas2 and protospacer (75 nM each). In these conditions, the quenching effects of spermidine on band 4 intensity were nearly the same with both plasmids (Fig. 5B). Next, we submitted to denaturing gel electrophoresis reaction samples incubated for 60 min in the presence of excess Cas1–Cas2:protospacer complex. Spermidine inhibited integration at all off-target sites on either pF0 or pF1 (Fig. S6). Finally, protospacer integration was assayed in the presence of both a fixed concentration of the CRISPR-containing plasmid pF0 and various concentrations of the CRISPR-free plasmid pF1. Inhibition by pF1 of protospacer integration at the CRISPR locus of pF0 could be detected only when spermidine was absent (Fig. 6). All these results indicated that spermidine favors the binding of Cas1–Cas2 at the CRISPR locus and/or disfavors that at off-target sites.

In Type I CRISPR systems, protospacers are always produced close to a protospacer adjacent motif (PAM) in the alien DNA (48, 49). To examine a possible effect of spermidine on the inte-

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**Figure 4. Effect of mono- or multivalent cations on the rate of protospacer integration into CRISPR plasmid pF0.** Reaction mixtures contained 7.5 nM protospacer P1 (Fig. 1), 7.5 nM Cas1–Cas2, 40 nM IHF, 7.5 nM supercoiled plasmid pF0, and the indicated concentrations of spermidine (A), spermine (B), putrescine (C), cadaverine (D), lysine (E), KCl (F), MgCl₂ (G), or CaCl₂ (H). Incubation time was 120 s. Restricted samples were analyzed by native agarose gel electrophoresis as explained in the legend to Fig. 1. Integration rates ([P]/([E] × t)) are plotted on the ordinate, where [E] is the total Cas1–Cas2 concentration in the assay (7.5 nM), t is the incubation time (120 s), and [P] is the concentration of protospacer integrated at the CRISPR locus (band 2 + band 3 on the agarose gel, red) or outside this locus (band 4, cyan). Insets show the deduced specificity index, defined as the ratio between the integration rates at, and outside, the CRISPR locus. To facilitate comparison of results, the same ordinate scale was used in all panels. Mean and range of two independent experiments are shown.
Cas1–Cas2 integrase and spermidine

Figure 5. Rates of off-target protospacer integration into CRISPR plasmid pF0 or non-CRISPR plasmid pF1. Reaction mixtures contained 7.5 or 75 nM Cas1–Cas2 + stoichiometric protospacer P1 (Fig. 1), 40 nM IHF, 7.5 nM supercoiled plasmid pF0 (magenta) or pF1 (green), and the indicated concentrations of spermidine. Restricted samples were analyzed by native agarose gel electrophoresis as explained in the legend to Fig. 1. Integration rates ([P]/([E] x t) x 10^3 (s^-1)) are plotted on the ordinate, where [P] is the concentration of protospacer integrated outside the CRISPR locus, [E] is the total Cas1–Cas2 concentration in the assay, and t is the incubation time (120 s). A, reaction with 7.5 nM Cas1–Cas2, B, reaction with 75 nm Cas1–Cas2. Mean and range of two independent experiments are shown.

Figure 6. Inhibition by non-CRISPR DNA (pF1) of protospacer integration at the CRISPR locus of plasmid pF0. Reaction mixtures contained 7.5 nM protospacer P1 (Fig. 1), 7.5 nM Cas1–Cas2, 7.5 nM supercoiled plasmid pF0, either 0 or 2 mM spermidine, and the indicated concentrations of supercoiled non-CRISPR plasmid pF1. IHF was present in excess (800 nM) of the highest pF1 concentration used. Restricted samples were analyzed by native agarose gel electrophoresis as explained in the legend to Fig. 1. Integration rates ([P]/([E] x t) x 10^3 (s^-1)) are plotted on the ordinate, where [E] is the total Cas1–Cas2 concentration in the assay (7.5 nM), t is the incubation time (120 s), and [P] is the concentration of protospacer integrated at the CRISPR locus of pF0 (red bars) or outside this locus (in both pF0 and pF1, cyan bars). Mean ± S.D. of three independent experiments are shown.

**Improvement by spermidine of the canonical integration reaction specificity depends on the presence of both IHF and a supercoiled DNA target**

Combined roles of polyamines and IHF have been described in several DNA-integrase systems (37, 50–52). On the other hand, IHF is an important player in the integration reaction at an *E. coli* CRISPR locus (12). We therefore investigated whether IHF was necessary to the actions of spermidine described above.

If IHF was omitted, polyamine addition caused a sharp decrease in the integration rate at the CRISPR locus of pF0 (Fig. 7A). This behavior markedly contrasted with the 1.5-fold stimulatory effect of the polyamine we observed in the presence of
and 7. In another series of protospacer integration assays, we wanted to evaluate the importance of the supercoiling of our target DNA in the effect of spermidine. At this end, we used HindIII-digested pF0 as the acceptor DNA. After incubation and prior to electrophoresis, the linearized plasmid was further restricted with KpnI providing DNA fragments A and B. In the presence of spermidine, the index varied linearly, reaching a value of 36 at 160 nM IHF. The results in Fig. 8 also indicated that such a large excess of IHF did not uncover cryptic sites for noncanonical integration reactions.

Another question was to know how much the spermidine effect we observed depended on the IHF concentration. Fig. 8 shows the results of protospacer integration assays in which IHF was varied from 0 to 160 nM, with 0 or 2 mM fixed spermidine concentration. Whatever the absence or presence of spermidine, the rate of specific integration increased with IHF concentration, reaching a plateau at 20–40 nM IHF. This behavior suggested that the IHF concentration we generally adopted in our experiments (40 nM), well in excess over the concentration of the CRISPR locus (7.5 nM), was sufficient to saturate the IHF-binding site in the CRISPR leader DNA. In the presence of the polyamine, the specificity index reached a maximum of ~300 at 40 nM IHF and remained constant beyond. In the absence of spermidine, the index varied linearly, reaching a value of 36 at 160 nM IHF. The results in Fig. 8 also indicated that such a large excess of IHF did not uncover cryptic sites for noncanonical integration reactions.

In another series of protospacer integration assays, we wanted to evaluate the importance of the supercoiling of our target DNA in the effect of spermidine. At this end, we used HindIII-digested pF0 as the acceptor DNA. After incubation and prior to electrophoresis, the linearized plasmid was further restricted with KpnI providing DNA fragments A and B. In the presence of spermidine and in the absence of polyamine, the rate of protospacer integration at the CRISPR locus was 1.8-fold lower than that measured with the supercoiled plasmid. This rate slightly increased upon addition of submillimolar concentrations of spermidine and decreased at concentrations higher
than 2 mM (Fig. S10). The rate of integration outside the CRISPR locus monotonically dropped as a function of the addition of polyamine. Overall, upon spermidine addition, the specificity index with the linearized DNA increased by a factor of 2.3-fold at the maximum, a factor markedly smaller than the improvement obtained with supercoiled DNA (∼30-fold).

The linearized plasmid was also studied in the absence of IHF (Fig. S10). No reaction occurred at the CRISPR locus, in agreement with a previous report (12). Off-target integration events still occurred despite the lack of IHF and were reduced by spermidine. We concluded that, in the presence of spermidine, DNA supercoiling and IHF presence cooperate to optimally drive the Cas1–Cas2 integration reaction in direction of the CRISPR locus.

Discussion

Here we followed foreign DNA (protospacer P1) integration in a supercoiled plasmid DNA by Cas1–Cas2 integrase. The targeted plasmid DNA we used (pF0, 3 kbp) harbors a minimal canonical CRISPR array composed of 62 nucleotides of the leader region and a single 28-nucleotide repeat. Noncanonical spacer integration reactions occurred, however, outside this canonical CRISPR array. Off-target and target integration events could be distinguished and quantitated.

It was already shown that the balance between target and off-target site reactions strongly depends on the presence of IHF (12, 27, 31). We show here that spermidine or spermine markedly potentiates this effect of IHF. With protospacer P1 as the substrate and CRISPR plasmid pF0 as the target, the ratio between the rates of protospacer integration events at and outside the CRISPR locus increased from 1.0 to 13.5 upon addition of saturating IHF. If spermidine (2 mM) was also added, this ratio rose to more than 300. We also observed that, in the presence of IHF, reduction of the off-target integration rate by spermidine was more pronounced if CRISPR-containing plasmid pF0 was used as the acceptor rather than the CRISPR-free plasmid pF1 (Fig. 5). This result suggested that, under our experimental conditions (i.e. 7.5 mM of each supercoiled plasmid pF0, Cas1–Cas2 integrase, and 33-bp protospacer), recruitment of Cas1–Cas2 at the CRISPR locus on pF0 in the presence of spermidine limited the free concentration of the integrase available to bind off-target sites. Moreover, inhibition of protospacer integration at the CRISPR locus of pF0 upon addition of pF1 could only be detected if spermidine was absent (Fig. 6). Therefore, it was likely that, in the presence of IHF, spermidine addition had shifted the balance between bindings at and outside the CRISPR locus at the benefit of the canonical integration site. These results raised the possibility that, in vitro, polyamines are essential partners of the CRISPR machinery to avoid unspecific integration reactions and guarantee overall genomic integrity.

This behavior is reminiscent of spermidine action on the accuracy of type II restriction endonucleases. In vitro studies established suppression by the polyamine of cleavage at degenerate nonsymmetrical sites, whereas improving cleavage at specific sites (53, 54). In vivo, restriction cleavage errors seem to be rare, implicating that fidelity of the endonuclease is high under physiological conditions, possibly through the presence of polyamines.

In vivo, protospacers are always produced close to a PAM motif in the alien DNA (48, 49). When we added a PAM consensus sequence (CTT) at one 3’-end of our model protospacer, integration only occurred at the leader-proximal end of the repeat (Fig. S7). Moreover, only the nonextended 3’-end reacted with the targeted repeat. These observations suggested that those unmatured protospacers that may be produced in vivo bind Cas1–Cas2 in a such way that half-site integration only occurs at the leader-repeat junction thanks to the nonextended 3’-end. To obtain full-site integration, maturation of the other protospacer end is required. In our work, using the simplified experimental system consisting of supercoiled DNA, extended protospacer and Cas1–Cas2 integrase, this maturation was not detectable, even in the presence of spermidine. It is likely that, in vivo, trans-action of additional nuclease(s) is involved (18, 21, 23, 55).

Spermidine is a binder of nucleic acids (57). It neutralizes the electric charge of DNA. Polyamines are often important in enzyme systems where distant DNA segments have to come to interaction (58). This is the case in the CRISPR integrase reaction where protospacer and target DNA have to be in contact. Possibly, spermidine disfavors electrostatic recruitment of the protospacer:Cas1–Cas2 complex at both the target and off-target sites. On the other hand, major groove closure by polyamines (59, 60) could favor IHF-dependent bending at the correct target. As a result, formation of a competent reaction complex at the CRISPR locus would be maintained or reinforced despite charge neutralization of the whole DNA target.

We observed that stability of the reactive DNA:Cas1–Cas2:protospacer:IHF complex at the CRISPR locus after DNA charge neutralization by spermidine needed supercoiling of the target DNA. Additive positive effects of supercoiling and IHF binding have already been described in transcription, recombination, and transposition systems (61–63), although a synergistic effect of supercoiling on the affinity of IHF for its target sites could be discarded (61). In contrast, millimolar concentrations of spermidine inhibited Cas1–Cas2-dependent integration at noncanonical sites whatever the DNA substrate, supercoiled or linearized. We noted, however, that the yield of the nonspecific integration reactions with a supercoiled plasmid showed a significant rising peak at low concentrations of spermidine (0.15 mM). A similar dependence on spermidine concentration was recently documented in a study dealing with polyamine effects on in vitro gene expression (64). Using atomic force microscopy, the authors showed formation of a flower-like DNA conformation at the submillimolar polyamine concentration (0.3 mM) ensuring maximum gene expression. They concluded that the enhancement they observed resulted from parallel ordering of DNA accompanied by a limited decrease in the negative charge of dsDNA. Note, however, that the atomic force microscopy experiments were performed in low ionic strength conditions (10 mM Tris-HCl). Possibly, the peak in off-target Cas1–Cas2 activity we observed at submillimolar spermidine resulted from similar DNA conformational modifications in the supercoiled DNA target. The question thus arises, what is the concentration of free spermidine in the bacterium?

Mutants of spermidine metabolism in bacterial cells have been obtained (65–67). In particular, an E. coli mutant deficient
in all genes involved in polyamine biosynthesis could be constructed (68). This mutant has no essential requirement for spermidine, but it shows retarded growth. The idea that can be drawn from all these studies is that disturbance of cellular spermidine has pleiotropic consequences because of the many putative roles of polyamines in the reactions involving nucleic acids (nucleic acid biosynthesis and structure, protein biosynthesis, λ phage integration...). Estimation of the concentration of free spermidine in the cell is still an imposing problem. Total amounts of spermidine in E. coli are reported around 5 mM (57, 71, 72). In vitro equilibrium dialysis and NMR experiments indicated that polyamines generally are not attracted by well-defined sites on nucleic acids but rather interact with these polyelectrolytic molecules through nonspecific electrostatic interactions (73, 74). These interactions are not tight and, consequently, cellular distribution between free and complexed spermidine cannot be reliably measured in cell extracts. Presently, only simulations of spermidine distribution in a cell are available. Miyamoto et al. (71) estimated binding constants of polyamines for polynucleotides and ATP by chromatographic measurements in ionic conditions close to those that are presumed to occur in vivo (150 mM K+, 10 mM Mg2+). According to the simulations that followed, up to 90% of total spermidine should be bound to RNA and free spermidine plus DNA-bound spermidine would not exceed 3.6 ± 5.1% of the total polyamine. Thus, starting from a total spermidine cellular concentration of 7 mM as taken by Miyamoto et al. (71), free spermidine plus DNA-bound spermidine would not exceed 0.7 mM. If proven in vivo, such a small concentration of DNA-bound spermidine would challenge the relevance of many effects of polyamines on DNA that have been described in vitro. In actuality, nucleic acids are highly charged polyelectrolytes surrounded by counter-ions and, therefore, the cloud concentrations of cations, especially multivalent cations such as polyamines, are likely to be much higher in the close vicinity of nucleic acids than elsewhere in the cytoplasm (75). In addition to all, the cation–nucleic acid interactions in vivo are probably biased by the very crowded cellular environment (76) and the existence of basic nucleic acid-binding proteins. Altogether, these considerations make difficult any definition of free cellular spermidine concentration.

In conclusion, it is tempting to imagine that the presence of polyamines in vivo gives bacterial and archaeal cells the capacity to reduce off-target spacer integration events that otherwise would be deleterious to genome integrity. Spermidine is nearly ubiquitous (77). Spermine is absent in E. coli but present in many cells (77). The Cas1–Cas2 integrase is conserved in most known CRISPR systems, in bacteria as well as in archaea (78). In contrast, IHF is not universally present (32). In this study, we focused on the Type I-E CRISPR system of E. coli. Núñez et al. (13) showed that IHF binds to four different Type I-E or Type I-F CRISPR leader sequences from various E. coli strains, suggesting that the IHF role in spacer acquisition is conserved in all E. coli CRISPR systems. IHF also stimulates leader-proximal integration catalyzed by Type I-F Cas1–Cas2–3 integrase of Pectobacterium atrosepticum (79). In contrast, IHF is absent in numerous prokaryotes that contain active CRISPR-Cas systems, including Gram-positive bacteria and archaea (80). With Sulfolobus solfataricus, crude extract addition was shown to stimulate in vitro site-specific integration of a protospacer in the Type I-A CRISPR system of this archaea (21). This behavior was supposed to reflect the action of a cellular factor present in the extract. This factor is still unidentified. Surprisingly, Type II-A CRISPR systems from Streptococcus pyogenes and Enterococcus faecalis succeed in protospacer incorporation into a linear CRISPR DNA in vitro despite the absence of any host factor (28, 81). The 3D structure of several snapshots of Type II-A Cas1–Cas2 complex during integration indicated that the catalytic Cas1 subunit inserts an α-helix into the minor groove of the leader duplex (28). The location of this α-helix is conserved in other Cas1–Cas2 structures (26, 29), suggesting a general leader-recognition mechanism. Insertion of the helix requires DNA bending and minor groove widening (28). DNA-bending factors and/or polyamines that close the major groove of DNA while opening the minor groove are therefore expected to facilitate the insertion. It would be interesting to know whether polyamines are general cofactors of the fidelity of Cas1–Cas2 integrases whatever the availability or not of an IHF mimic.

### Experimental procedures

#### Cloning

The ihfa and ihfb genes were amplified from the chromosomal DNA of E. coli FB810 (a recA− derivative of BL21(DE3) (82)) using oligonucleotides IHFA-F and IHFA-R for ihfa, and IHFB-F and IHFB-R for ihfb (Table S1). Amplified ihfa was inserted between the BamHI and NotI sites of pACYCDuet-1 expression vector (Novagen) and amplified ihfb was inserted into the BgIII and Xhol sites of the resulting plasmid, to give plasmid pCMY-IHFA+β. DNA fragments harboring WT and mutant CRISPR loci were introduced between SacI and KpnI sites of pBluescript SK+. Their sequences are shown in Fig. S2.

#### Gene expression and protein purification

The Cas1 and Cas2 proteins were purified from E. coli FB810 as previously described (42). IHF was purified from FB810 cells transformed with plasmid pCMY-IHFA+β. This strain expressed both a native IHFβ subunit and an IHFα subunit containing an N-terminal His tag followed by a 3C protease cleavage site. Cells were grown at 18 °C for 27 h in 3 liters of autoinducible terrific broth medium (Formedium AIMT0210) containing 25 μg/ml of chloramphenicol. They were harvested by centrifugation (5,000 × g, 30 min) and resuspended in 170 ml of lysis buffer (20 mM Hepes-KOH, pH 7.5, 500 mM KCl, 5 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol) containing three complete EDTA-free protease inhibitor mixture tablets (Roche). Cells were disrupted by sonication (10 min, 4 °C) and debris was removed by centrifugation (35,000 × g, 20 min). The supernatant was applied on a Talon column (5 ml, Clontech) equilibrated in buffer A (20 mM Hepes-KOH, pH 7.5, 250 mM KCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol), then washed with buffer A containing 5 mM imidazole and eluted with a linear gradient from 0 to 250 mM imidazole in buffer A (1 ml/min, 3.5 mM/min). Fractions containing His6-tagged IHF were identified by SDS-PAGE analysis. They were pooled and dialyzed...
against buffer C (20 mM Hpes-KOH, pH 7.5, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) containing 150 mM KCl. The resulting sample (10 ml) was loaded on a HiTrap-Heparin HP column (5 ml, GE Healthcare) equilibrated in the same buffer, then washed with the same buffer and eluted with a linear gradient from 150 to 1000 mM KCl in buffer C (2.5 ml/min, 20 mM/min). Fractions containing His$_6$-tagged IHF were pooled (17.5 ml) and concentrated on a Vivaspin 20 (Sartorius) filtration tube to a volume of 400 µl. To remove the affinity tag, 45 µg of His$_6$-tagged 3C protease were added and the resulting sample was incubated at 4°C overnight. Finally, IHF heterodimer was separated from the protease and the affinity tag by chromatography on a Talon column (1 ml) equilibrated and eluted by gravity with buffer A. Purified IHF was stored in 20 mM Hpes-KOH, pH 7.5, 250 mM KCl, and 60% (v/v) glycerol at −20°C. SDS-PAGE analysis indicated that impurities in the IHF preparation were lower than 5%.

**Protospacer integration assays**

Integration assays were performed using fluorescent oligonucleotides labeled at their 5’-end with DY682 (shown in red in the figures) or DY782 (in green in the figures) (from Eurofins Genomics). In these oligonucleotides, the fluorescent dyes were covalently attached by (i) coupling a C6 amino linker to the 5’-end of the oligonucleotide by a phosphate bridge and (ii) reacting this amino group with an N-hydroxysuccinimide ester derivative of the dye.

The protospacer integration assay mixture (20 µl) contained 20 mM Hpes-KOH buffer, pH 7.5, 50 mM KCl, 5 mM EDTA, 1 mM DTT, 0.15% (v/v) Tween 20, and 200 µg/ml of BSA. Pre-anneled double-stranded protospacer molecules were first incubated with stoichiometric amounts of Cas1-Cas2 for 10–15 min at 22°C. The Cas1–Cas2:protospacer complex was then added to a sample containing plasmid DNA with or without IHF. The resulting mixture was preincubated at 22°C for 1 h and, next, pre-heated at 37°C for 10 min. The reaction was started by addition of MgCl$_2$ at a final concentration of 10 mM (5 mM in excess over the EDTA concentration). After additional variable incubation time at 37°C, the reaction was stopped by addition of EDTA (20 mM final) and SDS (0.8% (w/v) final). Plasmid DNA molecules were precipitated with ethanol, centrifuged, and lyophilized (not air-dried at room temperature, which results in partial denaturation of small DNA fragments (56)), dissolved in Thermo Fisher FastDigest buffer and digested by HindIII and KpnI restriction enzymes. These samples were analyzed by electrophoresis in 2% (w/v) native agarose gels containing 45 mM Tris borate, pH 8.3, and 1 mM EDTA, or in 8% (w/v) denaturing polyacrylamide gels containing 90 mM Tris borate, pH 8.3, 2 mM EDTA, 7 M urea, and 40% (v/v) formamide. Gels were scanned for fluorescence of DY682 and DY782 with a Li-Cor Odyssey CLx IR imaging system. This scanner operated with solid-state diode lasers that simultaneously provided light excitation at 685 and 785 nm.

ImageJ software was used to quantify the intensity of electrophoretic bands. To determine the concentrations of transformed products, it was assumed that total fluorescence signal in a lane corresponded to the initial protospacer concentration in the sample. Products on native agarose gels were quantitated using the DY782 (green) signal because it was less noisy than DY682 (red). Initial rates of integration were approximated by calculating $[P]/([E] \times t)$ at an incubation time $t$ of 2 min, where $[E]$ is the total Cas1–Cas2 concentration in the assay (7.5 nM), and $[P]$ is the concentration of integrated protospacer. This approximation was justified by the fact that the amount of integrated protospacer at or outside the CRISPR locus increased roughly linearly with time during the 2-min incubation (see gray and blue curves in Fig. S3).

DY682-labeled size markers were prepared by PCR using pBSZ2 (42) as template and DY682-M13-uni and OCN400 as primers (marker of 96 bases), pBSZ2 as template and DY682-M13-uni and OCN443 as primers (125 bases), pBSZ4 (42) as template and DY682-M13-uni and M13-rev as primers (155 bases), or pBSZ3 (42) as template and DY682-M13-uni and M13-rev as primers (188 bases). The PCR-amplified DNA fragments were purified from agarose electrophoretic bands using the NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel.

**Author contributions**—P. P. and S. B. conceptualization; P. P. and S. B. supervision; P. P., C. M., and S. B. investigation; P. P. and C. M. methodology; P. P. and S. B. writing—original draft; P. P., C. M., and S. B. writing—review and editing.

**Acknowledgments**—We gratefully acknowledge Hannu Myllykallio for access to the Li-Cor Odyssey CLx imaging system and Michel Frohman for early contributions to this work.

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