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The DNA Replication Fork Blocked at the *Ter* Site May Be an Entrance for the RecBCD Enzyme into Duplex DNA

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In *Escherichia coli*, eight kinds of chromosome-derived DNA fragments (named Hot DNA) were found to exhibit homologous recombinational hotspot activity, with the following properties. (i) The Hot activities of all Hot DNAs were enhanced extensively under RNase H-defective (*rnh*) conditions. (ii) Seven Hot DNAs were clustered at the DNA replication terminus region on the *E. coli* chromosome and had Chi activities (H. Nishitani, M. Hidaka, and T. Horiuchi, Mol. Gen. Genet. 240:307–314, 1993). Hot activities of HotA, -B, and -C, the locations of which were close to three DNA replication terminus sites, the *TerB*, *-A*, and *-C* sites, respectively, disappeared when terminus-binding (Tau or Tus) protein was defective, thereby suggesting that their Hot activities are termination event dependent. Other Hot groups showed termination-independent Hot activities. In addition, at least HotA activity proved to be dependent on a Chi sequence, because mutational destruction of the Chi sequence on the HotA DNA fragment resulted in disappearance of the HotA activity. The HotA activity which had disappeared was reactivated by insertion of a new, properly oriented Chi sequence at the position between the HotA DNA and the *TerB* site. On the basis of these observations and positional and orientational relationships between the Chi and the *Ter* sequences, we propose a model in which the DNA replication fork blocked at the *Ter* site provides an entrance for the RecBCD enzyme into duplex DNA.

Homologous recombination on the chromosome is often uniform. However, in both procaryotes and eucaryotes, there are specific regions or sites, named hotspots, where homologous recombination occurs at a higher rate. DNA replication origin in procaryotes (phage) is one example (56). Another example is the *HOT1* site in *S. cerevisiae*, which has activity to stimulate recombination homologously in adjacent regions (54). Molecular mechanisms involved in enhancing homologous recombination are not fully characterized. Microscopically, there is a site where the homologous recombination of the surrounding region is stimulated. The Chi site is such a recombinational hotspot and was first identified in lambda phage (16, 32, 46). The Chi site enhances recombination not just in its immediate vicinity but even as far away as 10 kb (41, 42). Chi consists of an 8-bp specific sequence, 5′-GCTG GTGG-3′, distributed in *Escherichia coli* chromosomal DNA (one site per 5 to 15 kb on the average) (35, 45). RecBCD, which is a Chi-responsive enzyme, enters into duplex DNA, probably through a double-stranded (ds) break (*cos* site in the case of lambda phage) and moves on it with concomitant DNA degradation, and exonuclease activity of the enzyme seems to be modulated by Chi only when the enzyme approaches Chi from the correct side, the result being an enhancement of homologous recombination in the surrounding region (11, 12, 27, 39, 42, 44, 48, 49, 51, 55). In various analyses, the lambda phage system has been the most extensively used because in the *E. coli* system there are numerous Chi sites and an entrance for the enzyme on the circular chromosome has not been identified (7, 42).

Under RNase H-defective conditions, a *dnaA*- and *oriC*-independent replication system can function in *E. coli* (23, 38). We attempted to clone new replication origin(s) activated in *rnh* mutant *E. coli* cells. Whole chromosomal DNA digested with the *Eco*RI enzyme was ligated with the Km^r DNA fragment and transformed to the *rnh* mutant host. From the Km^r transformants, we obtained eight kinds of plasmid-like DNA, each of which contained a specific DNA fragment, termed Hot. Seven of the Hot DNAs (HotA through HotG) were located within a narrow DNA replication termination region (about 280 kb). Because Hot DNA could not be transformed into a mutant strain in which the Hot corresponding region on the chromosome had been deleted, the Hot DNA (though obtained as covalently closed circular [ccc] DNA) formed through excision from the host chromosome into which Hot DNA had once been integrated rather than through an autonomous replication. Consistent with this notion, Chi activity was present on all the Hot DNA so far tested and a Hot-positive clone without Chi activity has not been obtained, except for a DNA clone carrying the *dif* site (37). We then further analyzed eight kinds of Hot DNA on the *E. coli* chromosome and found that Hot activity of three of them (HotA-, -B and -C) was dependent on termination events and, in addition, at least one (HotA) was dependent on a Chi sequence present on the HotA DNA. From these results and positional and orientational relationships between the replication terminus (*Ter*) (17, 20) and the Chi sequence, we propose a model in which a ds break is introduced at the DNA replication fork impeded at the *Ter* sites. This break allows the RecBCD enzyme to enter the *E. coli* chromosome.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. All bacterial strains used were derivatives of *E. coli* K-12. W3110 (1), JM83 (53), plasmid pUC9 (53), and P1vir phage were from laboratory stocks. MIC1020 (24), kindly provided by M. Itaya, was used as the donor of *rnh399*::*cat* marker. M13mp10RF DNA and pHSG399, a Cm^r vector plasmid derived from pUC (50), were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). Two kinds of Km^r DNA fragments (7 and 1.4 kb long) were as described elsewhere (37).

Hot assay. Procedures used to measure the amount of ccc DNA recovered from the transformant with repeated Hot DNAs were as described elsewhere (37).

Other materials and procedures. Media, antibiotics, enzymes, reagents for DNA manipulation and general methods for DNA manipulations were as described previously (37).

Construction of *rnh tau* double mutants. W3110 (*rnh399*::*cat*) strain was constructed by P1 transduction of the *rnh399*::*cat* marker from the donor MIC1020 (*rnh399*::*cat*) to the recipient (W3110) and subsequent Cm^r selection. To this W3110 (*rnh399*::*cat*), each Hot DNA was transformed, and Km^r (Hot DNA) transformants were selected. To each transformant the *tau*$^+$ and *tau* mutant markers were transduced by P1 phage grown in the *tau*$^+$::Tn*3* (#43) (Tn*3* outside of the *tau* gene) and mutant *tau*::Tn*3* (#13) donor strains (28), respectively. A pair (*tau*$^+$ and mutant *tau*) of Ap^r transductants for each Hot DNA was prepared.

Construction of a strain carrying the Chi^0 mutation at the corresponding Chi$^+$ site of HotA DNA on the chromosome. First, an M13-HotA DNA (M13mp10RF DNA carrying the 4.3-kb HotA fragment at the *Eco*RI site) was constructed. An oligonucleotide containing the Chi^0 sequence at the corresponding Chi$^+$ site of the HotA DNA fragment was synthesized (see Fig. 4) and hybridized with the M13-HotA single-stranded DNA, and in vitro mutagenesis was done with a kit (oligonucleotide-directed in vitro mutagenesis system, version 2; Amersham) according to the protocol contained in the kit. After confirmation of the base change (that is, the appearance of a new *Bam*HI site) the HotA (Chi^0) DNA fragment was obtained from the M13-HotA (Chi^0) replicative form DNA, by *Eco*RI digestion, ligated with the Km^r fragment, and transformed into an *rnh*-mutant host strain in order to obtain Km^r transformants. Among them, a transformant, whose chromosome structure was HotA(Chi$^+$)-Km^r-HotA(Chi$^+$)-*TerB* (represented as Chi$^+$/Chi^0) was selected by *Bam*HI sensitivity assay for the two short DNA fragments covering the two Chi sites amplified through PCR, using two different sets of primers. From the transformant, Km^s segregants were selected, after cultivation in kanamycin- free Luria-Bertani (LB) broth, among which a strain carrying the Chi^0 allele in place of the original Chi$^+$ of HotA DNA was obtained. Using this HotA (Chi^0) strain as host, we transformed HotA (Chi$^+$) Km^r plasmid-like DNA and obtained transformants with a Chi$^+$/Chi^0 type of Chi allele. Conversely, with the HotA (Chi$^+$) strain as host, a ligation mixture containing HotA (Chi^0) DNA and a Km^r fragment was transformed and a Chi^0/Chi$^+$ type transformant was obtained. The same ligation mixture was used to transform a HotA (Chi^0) host, resulting in a Chi^0/Chi^0 type transformant. These transformants with all four combinations of Chi allele were used in experiments to investigate the Chi sequence dependency of Hot activity.

Construction of Chi^0/Chi^0 type transformants carrying an external Chi$^+$ sequence at the *Hin*dIII site between HotA DNA and *TerB* site on the chromosome. First, a unique *Hin*dIII site of vector plasmid pHSG399 (Cm^r) was destroyed by *Hin*dIII digestion, subsequent blunting, and ligation. Into the *Eco*RI site of this *Hin*dIII^r pHSG399 plasmid, the *Eco*RI *TerB* fragment which carried the Tn*3* transposon at the site (#41) between the *Hin*dIII and the *TerB* sites was recloned (see Fig. 7). Into a *Hin*dIII site of the resulting plasmid (pHSG399-*TerB* [#41]), a synthetic oligonucleotide containing a *Pvu*II-Chi$^+$-*Bal*I sequence was inserted and a plasmid containing *Hin*dIII^r and newly acquired *Pvu*II site was selected. The presence and orientation of the Chi sequence at the desired site were confirmed by PCR. A pair of orientation-isomeric Chi$^+$-containing plasmids was linearized by *Eco*RI digestion and transformed to a *recD* mutant by electroporation to obtain Ap^r transformants. Using PCR, we confirmed that these transformants acquired Tn*3* together with the Chi sequence on the chromosome from the linearized plasmid. The external Chi$^+$ and Ap^r(Tn*3* markers were then transferred into a recipient strain (*rnh399*::*cat*; HotA[Chi^0]-Km^r-HotA [Chi^0]) by P1 transduction. In the transductants, the majority were HotA(Chi^0)-Km^r-HotA (Chi^0)-external Chi$^+$-Tn*3*-*TerB* transductants (see structure in Fig. 7a); some transductants which acquired the original Chi$^+$ site of HotA from the donor *recD* mutant simultaneously with the external Chi$^+$ and Tn*3* markers (that is, HotA [Chi^0]-Km^r-HotA [Chi$^+$]-external Chi$^+$-Tn*3*-*TerB*) were used in the HotA assay as control strains.

RESULTS

Properties of Hot DNAs and their locations on the *E. coli* chromosome. In earlier work, we isolated and analyzed *Eco*RI DNA fragments from the *E. coli* chromosome with higher homologous recombinational (hotspot) activity (37). In summary, the whole *E. coli* chromosomal DNA was digested with *Eco*RI enzyme, ligated with the 7-kb Km^r fragment and introduced into the RNase H-defective (*rnh*) host (23) before Km^r transformants were collected. An explanation for use of the *rnh* mutant is given in the introduction. From these transformants, plasmid-like ccc DNA, the excisional product from repeated Hot DNAs on the *E. coli* genome (see the introduction), was extracted by the alkaline miniprep method (36). We found specific DNA, recovered as the ccc DNA form, in larger amounts than DNAs recovered from other clones. We classified these DNA fragments into eight groups, termed HotA to HotH DNA. Because the amount of ccc DNA recovered from the transformant depends on recombinational Hot activity, we called this method, Hot assay. Common properties shared by the eight kinds of Hot DNAs are (i) extraordinarily high Hot activities under RNase H-defective conditions (see Fig. 2) and (ii) Chi activities (except for HotE and HotF; see Fig. 1 legend) (37). Their locations on the physical map of the *E. coli* genome (5, 30) are shown in Fig. 1. Interestingly, seven of the Hot DNAs (HotA through HotG) were located within a narrow (about 280-kb) DNA replication terminus region with no particular selection. Figure 2 shows the Hot activity of HotA DNA. For comparison, we used a DNA fragment carrying *dif*, a *cis* site on the chromosome which acts as a resolving site from the dimer to the monomer form of the *E. coli* chromosome, aided by a *trans*-acting factor, XerC protein, in the same manner as for the Hot DNA fragment in the Hot assay (4, 8, 31). *dif*-XerC is a typical, site-specific recombinational system which apparently functions at high efficiency. As shown in Fig. 2, the level of ccc DNA recovered from either type of transformant was very high; DNA fragments produced from *Eco*RI digestion of ccc DNA (which were extracted from 1.5 ml of overnight culture) were visible by ethidium bromide staining after agarose gel electrophoresis. In a separate experiment, we estimated the

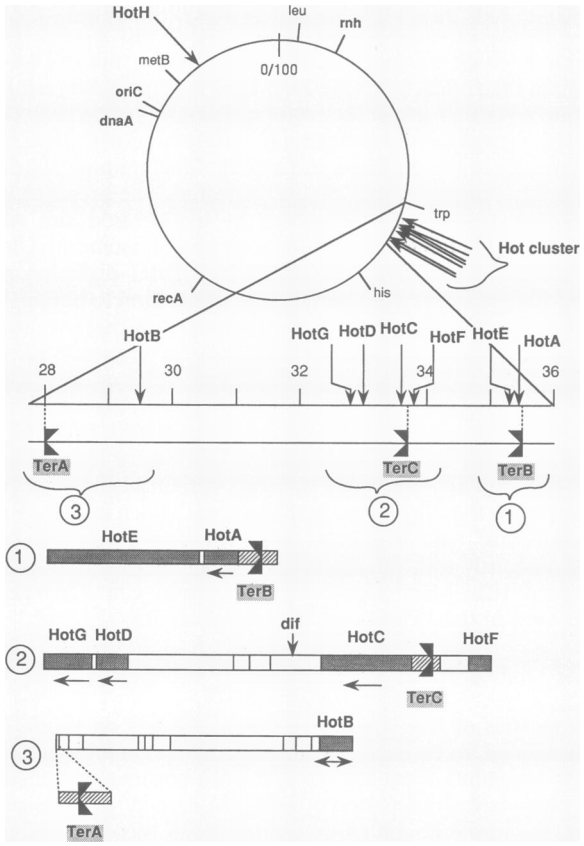

FIG. 1. Locations and Chi activity of Hot DNA fragments on the *E. coli* chromosome. (This figure is identical to Fig. 1 in our previous report [37] except for the Chi activities and orientations.) Locations of Hot DNA fragments on the *E. coli* circular map (2) are shown above and the three expanded *Eco*RI restriction maps, with three *Ter* sites (paired triangles) and seven Hot DNAs (■). The scale of map 3 is half that of the others. The lengths of the Hot fragments are as follows (in kilobases): A, 4.25; B, 8.3; C, 11.4; D, 4.25; E, 19.5; F, 3.0; G, 6.1; and H, 11.2. Chi activity and its orientation are shown by horizontal arrows; note the polarity of the Chi activity (Chi is active when the RecBCD enzyme moves in the direction indicated by the arrow). Chi activities of HotE and HotF fragments were not tested (35). Relevant markers, including *dif* sites, are also shown.

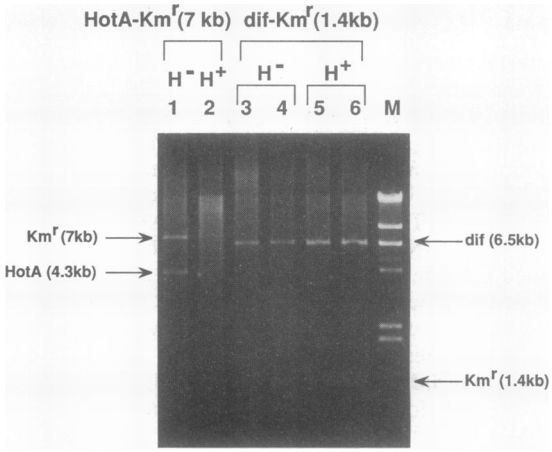

FIG. 2. Comparison of Hot activities of the HotA and *dif* DNA fragments. W3110 (wild-type) and W3110 (*rnh399*::*cat*) strains were transformed with HotA (4.25-kb) Km^r (7-kb) or *dif* (*dif*-carrying DNA fragment; 6.5-kb) Km^r (1.4-kb) plasmid-like DNAs. Overnight culture (Luria-Bertani broth plus kanamycin; 1.5 ml) of each transformant was subjected to Hot assay as follows. Plasmid-like DNA was extracted, digested with *Eco*RI, and analyzed by agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV irradiation. Lanes: 1, HotA Km^r DNA from *rnh* mutant host; 2, the same DNA as in lane 1, but from wild-type host; 3 and 4, *dif* Km^r DNA from two independent clones of *rnh* mutant host; 5 and 6, the same DNA as in lanes 3 and 4, but from two independent clones of wild-type host. H$^+$ and H$^-$ indicate that *rnh*$^+$ and *rnh* mutant strains, respectively, were used as the host, M indicates a DNA molecular marker (lambda phage DNA digested with *Hin*dIII) containing fragments of the following (kilobase) lengths: 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.5.

amount of recovered HotA DNA to be 20 to 30% of that of miniF DNA (data not shown). HotA activity, which depends on *recA*$^+$ (data not shown) and *rnh* mutation, is comparable to that of the *dif*-XerC system, which depends on the *dif* site (4, 31) but not on the *rnh* mutation. Thus, the efficiency of recombinational excision rate of repeated HotA DNAs is apparently high. Dependency of the Hot activity on *recBCD* genes could not be determined because the *rnh recBCD* double mutant is not viable (24).

Hot activities of some Hot DNAs are DNA replication termination event-dependent. In the terminus region, we and other groups identified six terminus (*Ter*) sites (15, 17, 18, 20, 40). A terminus site, which consists of a specific 22-bp sequence, has activity to block progression of the DNA replication fork from one, but not the opposite, direction (17, 20, 22). For example, *TerB* can inhibit only the clockwise, not the counterclockwise, replication fork (Fig. 1). To express fork-blocking activity, another protein factor, termed *Ter* binding protein, is required (19, 21, 28). The protein, which is coded in a gene called *tau* or *tus*, can bind at the *Ter* site, specifically, and the resulting *Ter* protein-*Ter* site complex has activity impeding the replication fork in a polar fashion, in vitro as well as in vivo (26, 33). Among six terminus sites, three of the innermost and most relevant *Ter* sites, *TerA*, *-B*, and *-C*, are shown in Fig. 1. Interestingly, all seven of these Hot DNAs are located between *TerA* and *TerB*. HotA and HotC DNA fragments are just next to the two *Eco*RI fragments carrying *TerB* and *TerC*, respectively. Such close location prompted us to search for a possible relationship between Hot and *Ter* activities. We measured Hot activities by using a mutant host strain carrying a *Ter*-binding protein-defective (*tau* or *tus*) mutation (21, 28) in addition to the *rnh* mutation. As shown in Fig. 3, HotB activity disappeared when the *tau* mutant was used as host, regardless of the length of Km^r fragments. On the other hand, HotG and HotD showed no *tau*$^+$-dependent Hot activities. Similar experiments were carried out for other Hot DNAs, and these results are summarized in Table 1. HotA, -B, and -C required the presence of *Ter*-binding protein for these activities; the termination event is probably essential for Hot activities. Hot activities of other DNAs (HotD, -F, -G, and -H) are *tau* independent; these might require another type of event which may be specific under *rnh* defective conditions.

HotA activity is Chi$^+$ dependent. As shown in Fig. 1, all Hot fragments had Chi activities (37). To examine the relationship between Hot and Chi activities, we used the HotA DNA fragment and determined its DNA sequence (data not shown). A Chi sequence was present at a site about 600 bp from the right end of the fragment, in the expected orientation (Fig. 4). In vitro mutagenesis was carried out to destroy the Chi activity.

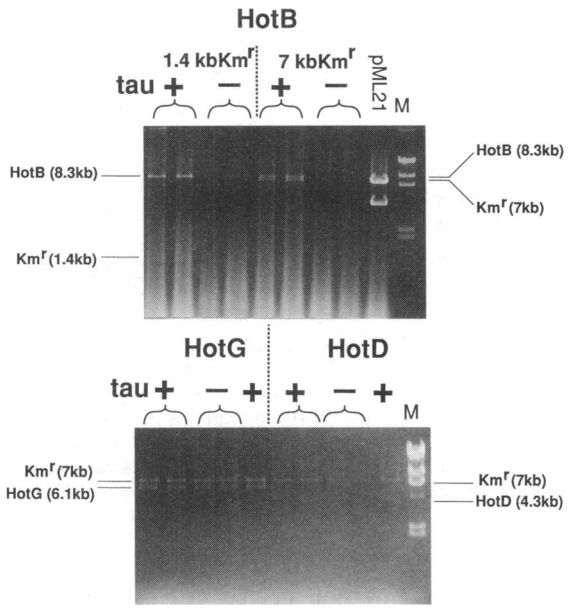

FIG. 3. Tau dependency of Hot activity. Each Hot Km^r (7- or 1.4-kb) plasmid-like DNA was transformed into the W3110 (*rnh399*::*cat*) strain, and Km^r transformants were selected. To each transformant, *tau*$^+$::Tn*3* (#44) or mutant *tau*::Tn*3* (#13) markers were transduced by P1 phage and two independent Ap^r transductants per recipient were obtained and subjected to Hot assay. Though HotB Km^r (7-kb) plasmid-like DNA contained an extra DNA fragment about 3 kb long, there was no apparent effect on Hot activity. In the lower panel, when W3110 (*rnh399*::*cat*) was used as host, the result was added in one lane (+). M, DNA molecular marker.

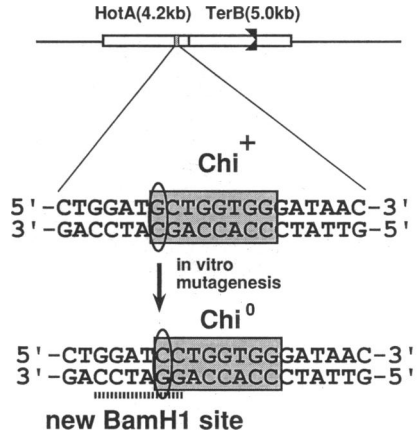

FIG. 4. In vitro mutagenesis from the Chi$^+$ sequence on the HotA DNA to (Chi^0). A Chi$^+$ sequence was found about 600 bp from the *Eco*RI site between the HotA and *TerB* fragments, and the G at the left end of the Chi$^+$ sequence was changed to a C by in vitro mutagenesis. This base change produced a new *Bam*HI restriction site. See Materials and Methods for details.

As shown in Fig. 4, the G · C pair at the left end of the Chi$^+$ sequence became the C · G pair (Chi^0); this alteration was reported to abolish Chi activity (43). This change produced a new *Bam*HI site; thus, *Bam*HI digestion could distinguish between the Chi$^+$ and Chi^0 alleles on the HotA DNA fragment to be tested. The wild-type HotA (Chi$^+$) plasmid-like DNA was introduced into the wild-type HotA (Chi$^+$) host; the chromosomal structure of HotA and its flanking region in the Km^r transformant is represented as Chi$^+$/Chi$^+$ in Fig. 5. The remaining three types of transformants, which carried possible combinations of two Chi alleles, were constructed, and the Hot activity of each transformant was measured (Fig. 5). After agarose gel electrophoresis, Southern hybridization was done, using the Km^r fragment as a probe to increase the sensitivity of the assay. HotA activity apparently depends on presence of the Chi sequence. When either of two repeated HotA DNA fragments carried the Chi^0 sequence, the HotA activity decreased to about one-half of that seen with the parental (Chi$^+$/Chi$^+$) transformant or less. The HotA activities in both Chi sequences with null mutations were reduced to 1/10 to 1/20 of the parental sequence activity. Thus, the active Chi sequence is essential to enhance the homologous recombination between repeated HotA DNAs. From the orientation of the Chi sequence it was deduced that the RecBCD enzyme must approach from the direction of the *TerB* toward the Chi sites (14).

TABLE 1. Hot activities in *tau*$^+$ and *tau* mutant strains

Hot DNA	Hot activity[a]	
	tau$^+$	*tau* mutant
A	+	−
B	+	−
C	+	−
D	+	±
F	+	++
G	+	+
H	+	+

[a] +, activity; ++, strong activity; ±, weak activity; −, no activity.

Next, we examined Chi dependency of HotA activity under conditions of an *rnh*$^+$ genetic background, the results of which are shown in Fig. 6. In this figure, the symbols (+ and 0) of the Chi allele indicate that repeated HotA DNAs have Chi$^+$ and Chi^0 markers, respectively. Under *rnh* mutant conditions, again about a 10-fold Chi$^+$-dependent stimulation of excisional recombination was observed. Moreover, this stimulatory effect by the Chi$^+$ allele was also observed when wild-type cells were used, albeit to a lesser (more than threefold on the average) but significant extent. These results suggest that in wild-type cells as well as *rnh* mutant cells, the RecBCD enzyme might enter into dsDNA via the *TerB* site, move on the DNA, and be activated by the Chi site. Enhancement of HotA activity by Chi in the wild-type strain was too faint for visualization of DNA bands in the *rnh*$^+$ lane in Fig. 2.

ccc HotA DNA was not the product of multimeric DNA replicated in a rolling-circle fashion. Dabert et al. (9) reported that a plasmid that replicates by a rolling-circle mechanism accumulates a large amount of high-molecular-weight linear multimers if the plasmid contains the Chi$^+$ sequence. Thus, the possibility that Hot ccc DNA might also be produced by monomerization of such multimers amplified through rolling-circle type replication after rare excision of repeated (Chi$^+$) HotA DNAs had to be considered (8).

The following experiments excluded this possibility. As described in the preceding section, HotA activity of the Chi^0/Chi^0 type transformant was at a background level. We constructed new transformants (Fig. 7a) on the chromosome where the repeated Chi^0 HotA was present at the original site and a newly synthesized Chi$^+$ oligonucleotide was inserted into a *Hin*dIII site located between the HotA DNA and the *TerB*

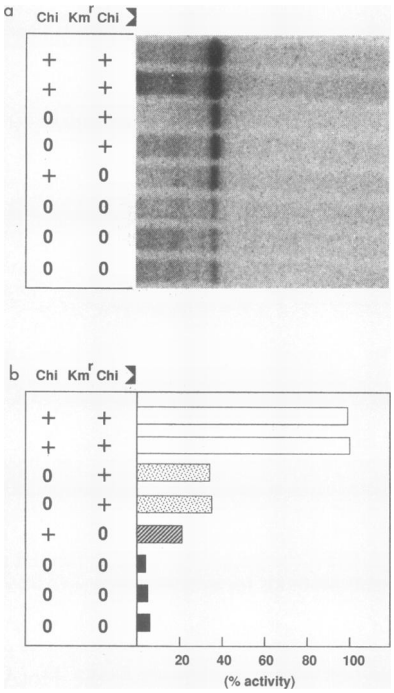

FIG. 5. Chi$^+$ dependency of HotA activity. A transformant with the chromosomal structure represented by HotA (Chi$^+$)-Km^r-HotA (Chi$^+$) (that is, Chi$^+$/Chi$^+$) was constructed by transformation of ccc HotA (Chi$^+$) Km^r DNA to the W3110 (*rnh399*::*cat*) host strain. Other transformants with three Chi allele combinations, Chi$^+$/Chi^0, Chi^0/Chi$^+$, and Chi^0/Chi^0, were also constructed (see Materials and Methods). DNA samples extracted from these strains (under alkaline conditions) were digested with *Eco*RI, analyzed by agarose gel electrophoresis, and transferred to nylon membrane, and DNA-DNA hybridization experiments were carried out with radiolabelled Km^r DNA fragment as a probe. The Chi allele of each Km^r transformant is shown on the left. (a) Autoradiography of the hybridization experiment, indicating Hot activities. (b) Graph of the radioactivity counts of each band in panel a, corresponding to the Km^r DNA fragment. The highest activity level was adjusted to 100%, and the other activities are relative.

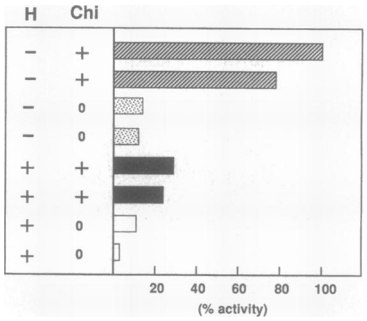

FIG. 6. Dependency of HotA activity on Chi sequence in wild-type cells. Experiments were carried out as described in the legend of Fig. 5. Alleles of the *rnh* gene (H) and the Chi site (Chi), are indicated on the left. The RNase H-defective strain used was the same as for Fig. 5, and the wild-type strain used was W3110. The Chi allele symbols indicate that both HotA DNAs in the transformants carried the Chi$^+$ (+) or Chi^0 (0) allele.

site, in both orientations. The HotA activities are shown in Fig. 7. In the case where both repeated HotA DNAs were Chi^0, the HotA activity was restored when an oligonucleotide with a Chi$^+$ sequence was inserted at the site between the HotA fragment and the *TerB* site in the same orientation as that of the original Chi$^+$ sequence. The level of HotA activity recovered by the external Chi$^+$ sequence was similar to that of the directly repeated HotA DNAs with Chi^0/Chi$^+$ configuration. We observed in another experiment that the presence of transposon Tn*3* (5.0 kb) at a site between HotA DNA and *TerB* sites had little effect on HotA activity (data not shown). Thus, a high rate of recovery of ccc HotA DNA did not require the Chi$^+$ sequence on the HotA DNA fragment itself. All or the majority of the ccc HotA DNA is, therefore, formed through excisional recombination rather than through a rolling-circle type replication.

DISCUSSION

Efficient excision of the repeated HotA DNA from the *E. coli* chromosome by homologous recombination apparently depends on a properly oriented Chi sequence present on or near the HotA DNA, since either mutational destruction of the Chi sequence or inversion of the external Chi sequence reduced Hot activity considerably (Fig. 5 and 7). The data on Chi-dependent HotA recombination in *E. coli* are consistent with results obtained through a thorough analysis of Chi, using the lambda phage system (27); however, there is a distinction between the two systems. In vitro, to enter duplex DNA RecBCD, a Chi-responsive enzyme, requires a ds break. In lambda phage, the *cos* site provides the entrance site for RecBCD (27). In *E. coli* also, RecBCD (Chi)-dependent transductional and conjugational recombinations were found, and dsDNA ends are present in both (13). However, on the *E. coli* circular chromosome, such a site has not been identified (42), though several RecBCD-mediated recombination phenomena have been noted. With regard to the entrance site for the RecBCD enzyme participating in HotA recombination, the most likely position is the DNA replication terminus, *TerB*, or a nearby site (17, 20). This is deduced from the finding that *TerB* locates at a satisfactory position for entrance of RecBCD (Fig. 4 and 7) and that a *tau* gene product, termination protein, is essential for HotA activity (Table 1). The termination event itself may even be required for HotA excisional recombination.

At the *TerB* site, the replication fork traveling clockwise, but not counterclockwise, on the circular chromosome (Fig. 1) is specifically impeded and a Y-shaped DNA molecule is formed, as shown in Fig. 8. In the *Ter*-binding-protein-deficient (*tau*) mutant, none of the Y-shaped molecules can be detected, because a termination reaction does not occur (28). On the other hand, in *rnh* mutant cells, a larger amount of the Y-form DNA molecule than in wild-type cells accumulates (17, 28). In *rnh* mutant cells, additional *oriC*- and *dnaA*-independent DNA replications initiate at new origins (23, 38). Because the two most active origins were located in the termination region (though the precise sites have not been identified [10]), the DNA replication fork starting at alternative origins is blocked immediately at one of the nearby *Ter* sites and the Y-shaped molecule accumulates. The order of the amounts of the Y-form molecule accumulated at *Ter* sites in these strains is *rnh* mutant > wild type > *tau* mutant (17, 28) and parallels the order of HotA activities in these strains. Thus, the DNA replication fork arrested at *TerB* may provide an entrance site for the RecBCD enzyme. As the ds break is probably required for entrance of the enzyme into duplex DNA (27, 47), the most likely event occurring at the junction of the Y-form molecule is the introduction of ds breaks, probably by nicking at a single-

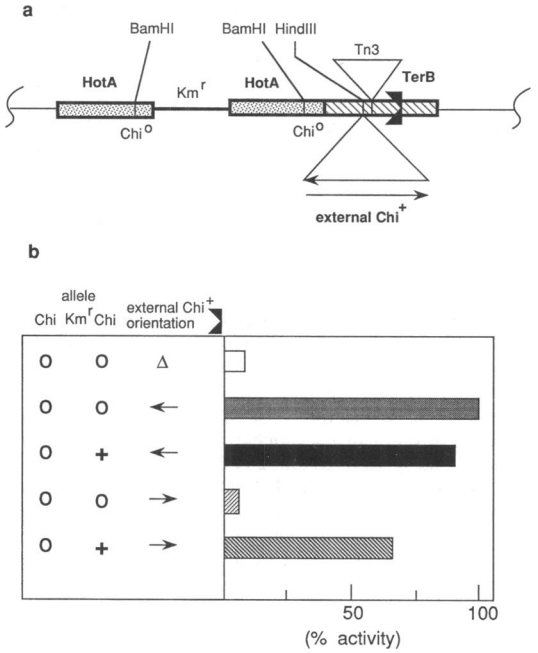

FIG. 7. Reactivation of Hot activity of Chi^0 HotA DNA by external, properly oriented Chi^+ sequence. (a) Chromosomal structure of the HotA transformant tested. A Chi^0/Chi^0 type of HotA transformant, which was derived from a W3110 *rnh* mutant, was made, and a synthesized oligonucleotide with Chi^+ sequence was inserted into a *Hin*dIII site located between the HotA DNA and *TerB* site on the chromosome, in both orientations. The resulting strains were subjected to Hot assay. Tn*3* was used as a selective marker when the synthesized Chi sequence was introduced into the host chromosome. (b) Hot activity of strains with the chromosome carrying external Chi^+ sequence. Δ, no insertion at the *Hin*dIII site; arrow, orientation of external Chi^+ sequence. When RecBCD travels through Chi^+ in the direction of the arrow, the enzyme is activated. Experiments were carried out as described in the legend of Fig. 5.

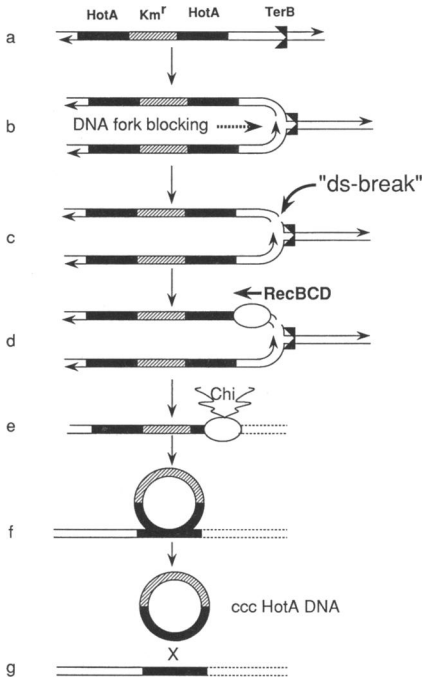

FIG. 8. A putative model for enhancement of excisional recombination between repeated HotA DNAs. (a) Chromosomal structure of a HotA DNA transformant, in which a Km^r fragment is located between repeated HotA DNAs. (b) When the DNA replication fork proceeds from left to right (this situation can occur under *rnh* defective conditions), the fork is blocked at the *TerB* site, and resulting Y-shaped molecules accumulate, the most in the *rnh* mutant, fewer in the wild type, and none at all in the *tau* mutant. (c) A ds break is introduced, probably by nicking at a single-stranded DNA complementary to the newly synthesized lagging strand. (d) The RecBCD enzyme enters the duplex DNA through the ds break and travels to the Chi site, with concomitant degradation of the newly synthesized dsDNA molecule by the exonucleolytic activity. (e) The Chi sequence modulates the exonucleolytic activity. (f and g) The resulting enzyme stimulates excisional homologous recombination between repeated HotA DNAs, resulting in production of a ccc HotA Km^r DNA molecule (11, 12, 49).

stranded DNA complementary to the newly synthesized lagging strand, as shown in Fig. 8. Bierne et al. (3) found that in a hybrid (M13ori + ColE1ori) plasmid, the *TerB* site was a deletion hotspot, thereby suggesting that the ds break at or very close to the *TerB* site might initiate the illegitimate recombinational event. The subsequent recombination process after RecBCD entrance through the Y-shaped DNA can be explained by the model proposed by Smith (42). In wild-type cells, enhancement of HotA activity is more suppressed than in *rnh* mutant cells but is significantly stronger than in *tau* mutants or the Chi^0 host (Fig. 3 and 6, Table 1, and data not shown). Consistent with this notion, Louarn et al. (34) observed, independently, that in the terminus region of a wild-type chromosome, homologous recombination occurred between repeated DNAs at a higher frequency than in other regions.

Several proposed models for molecular mechanisms of general recombination (for an example, see reference 52) have been constructed on the basis of the assumption that a double chain break occurs on the DNA duplex molecule, creating the initiation site for recombination, but how and where the break occurs have remained unknown, especially under replication-permitting conditions. Our present work strongly suggests that *Ter* is an entrance site for the RecBCD enzyme and might be an initiation site of other recombination pathways. In addition to the *Ter* site, the DNA replication fork, halted either at specific or nonspecific sites (transiently) or in a region damaged by physical and chemical reagents, would provide another entrance site for the RecBCD enzyme; in the former case, low but detectable levels of RecBCD-mediated recombination would be induced everywhere on the chromosome and in the latter case, the RecBCD enzyme would take care of recombinational repair of the damages. These speculations are consistent with the observations reported by Stahl et al. (47) that a Chi site on the lambda genome that was silent (that is, inactively oriented) under nonpermissive replication conditions was activated under replication-permitting conditions. From these data, they deduced that the replication fork might be the entrance for the RecBCD enzyme. In *rnh* mutant cells, if the ds break which would occur frequently at the *Ter* sites is repaired by the RecBCD enzyme, then a defect in either of the *recBC* genes might lead to cell death owing to an incomplete recombinational repair.

In another Hot group (HotD to HotG), Hot activity is *rnh*-mutant dependent but termination independent. Thus, it might require another event induced under *rnh* defective conditions which allow a new origin(s) to function. Thus, the second class of Hot DNA might depend on the initiation event

at new origins for Hot activity. If so, the initiation process as well as termination may provide entrance for RecBCD. Indeed, in the T4 phage one of the recombinational hotspots was found to have a replication origin (56). We are searching for unidentified origins activated under *rnh* mutant conditions, around Hot sites belonging to the second class.

HOT1, a recombinational hotspot in *S. cerevisiae*, has properties similar to the Hot described here. (i) Both have activity enhancing recombination of the downstream homologous region (25). (ii) Both require two components: *Ter* and Chi sites in *E. coli* and E (enhancer) and I (initiator) in *S. cerevisiae* (54). (iii) One of the two components in both cases has DNA fork-blocking activity. Brewer et al. (6) and our group (29) independently found that on yeast chromosomes, DNA replication fork-blocking activity located at the site almost identical with the E element. Thus, in *S. cerevisiae* as well as in *E. coli* the Y-form DNA structure at the fork-blocking site may be an entrance for yeast recombination enzyme(s).

Since the recombination system is active and the components are well characterized, a reconstruction of recombination in vitro is being considered.

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