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Characterization of the Functional Sites in the *oriT* Region Involved in DNA Transfer Promoted by Sex Factor Plasmid R100

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We have previously identified three sites, named *sbi*, *ihfA*, and *sbyA*, specifically recognized or bound by the TraI, IHF, and TraY proteins, respectively; these sites are involved in nicking at the origin of transfer, *oriT*, of plasmid R100. In the region next to these sites, there exists the *sbm* region, which consists of four sites, *sbmA*, *sbmB*, *sbmC*, and *sbmD*; this region is specifically bound by the TraM protein, which is required for DNA transfer. Between *sbmB* and *sbmC* in this region, there exists another IHF-binding site, *ihfB*. The region containing all of these sites is located in the proximity of the *tra* region and is referred to as the *oriT* region. To determine whether these sites are important for DNA transfer in vivo, we constructed plasmids with various mutations in the *oriT* region and tested their mobilization in the presence of R100-1, a transfer-proficient mutant of R100. Plasmids with either deletions in the *sbi-ihfA-sbyA* region or substitution mutations introduced into each specific site in this region were mobilized at a greatly reduced frequency, showing that all of these sites are essential for DNA transfer. By binding to *ihfA*, IHF, which is known to bend DNA, may be involved in the formation of a complex (which may be called *oriT*-some) consisting of TraI, IHF, and TraY that efficiently introduces a nick at *oriT*. Plasmids with either deletions in the *sbm-ihfB* region or substitution mutations introduced into each specific site in this region were mobilized at a reduced frequency, showing that this region is also important for DNA transfer. By binding to *ihfB*, IHF may also be involved in the formation of another complex (which may be called the TraM-IHF complex) consisting of TraM and IHF that ensures DNA transfer with a high level of efficiency. Several-base-pair insertions into the positions between *sbyA* and *sbmA* affected the frequency of transfer in a manner dependent upon the number of base pairs, indicating that the phasing between *sbyA* and *sbmA* is important. This in turn suggests that both *oriT*-some and the TraM-IHF complex should be in an appropriate position spatially to facilitate DNA transfer.

Conjugal DNA transfer promoted by bacterial sex factor plasmids involves a series of reactions, such as nicking at the origin of transfer, *oriT*, unwinding of the double-stranded DNA from the nick to produce single-stranded DNA, and transfer of the single-stranded DNA into the recipient cell (for a recent review, see reference 11). R100 is a sex factor plasmid and encodes several proteins, such as TraI, TraY, and TraM, essential for conjugal DNA transfer (for a review, see reference 11). Of these Tra proteins, the TraI protein (DNA helicase I) possesses the *oriT*-specific nicking activity (17, 20, 24, 27). TraY as well as integration host factor (IHF) enhances the nicking activity of TraI (17). Recognition and binding sites of these proteins have been identified and mapped in vitro in the proximal region (called the *oriT* region) of the *tra* genes required for conjugation promoted by R100; *sbyA* and *ihfA* were identified as the binding sites of the TraY protein and IHF, respectively (16, 18), and *sbi* was identified as the recognition site of the TraI protein (13). The TraM protein, which is a membrane protein (3), specifically binds to a region consisting of four sites (*sbmA* to *sbmD*) and is supposed to anchor the *oriT* region to the membrane (1). Two of the four TraM-binding sites, *sbmC* and *sbmD*, overlap the promoter for *traM*, and TraM represses its own expression by binding to these two sites (2). In the middle of the *sbm* region, there exists another IHF-binding site, *ihfB* (16). IHF binding to *ihfB* is supposed to form an active complex responsible for DNA transfer (2).

All of the sites recognized or bound by Tra and IHF proteins occupy in an array the *oriT* region almost entirely. These sites have been identified and mapped in vitro, but it is not known whether these sites are functionally important for DNA transfer in vivo. In this communication, we report the results of analysis of the transfer abilities of plasmids with mutations introduced in the *oriT* region, demonstrating that all the specific sites in the *oriT* region are important for DNA transfer.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains used are listed in Table 1. JM109 and BW313 were used for the site-directed mutagenesis described by Kunkel (22). DH1Rif is a rifampin-resistant mutant of DH1 that occurred spontaneously in the DH1 culture. NM554 and DH1Rif were used in the mating experiments described below. JE177 harbored plasmid R100-1.

The plasmids used were pSI87-B19, pSI87-ES3, pSI87-XE2 (19), pHSG299 (31), pUC119 (32), and R100-1 (9). R100-1 is a transfer-proficient mutant of R100 with a frameshift mutation within fertility inhibition gene *finO* and thus is transferred at a frequency about 1,000 times higher than that of R100 (34). Plasmid pHSG299 is a derivative of a high-copy-number plasmid, pUC19, and carries the kanamycin resistance gene instead of the ampicillin resistance gene. This plasmid was chosen in the present study because the cells carrying this plasmid form distinct colonies on kanamycin-containing plates, which do not allow residual growth of kanamycin-sensitive cells. pABO plasmids, which are pHSG299 derivatives carrying a DNA segment of R100, were constructed as described below. Plasmid DNA was prepared by the alkaline-sodium dodecyl sulfate method (28). The crude lysis method (23) was used to check for the presence of various plasmids.

Media, enzymes, and reagents. Luria-Bertani (LB) broth (25) was used for bacterial cultures. ϕ-Medium (34) and TSS medium (5) were used for transformation of the bacterial cells. LB agar plates which contained 1.5% (wt/vol) agar (Eiken) were supplemented with 0.2% (wt/vol) glucose and appropriate antibiotics or reagents when necessary for the selection of transconjugants and transformants. The antibiotics and reagents used were ampicillin (100 μg/ml; Wako),

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TABLE 1. *E. coli* strains used

Strain	Genotype or phenotype	Reference or source
JM109	F′ (*traD36 proAB lacI*^{q}ZΔM15)/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)	33
BW313	*hfr lysA dut-1 ung-1 thi-1 recA1 spoT1*	22
DH1	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA*	4
DH1Rif	DH1 Rif^r	This work
NM554	F$^-$ *araD139* Δ(*ara-leu*)*7679* Δ*lacX74 galU galK hsdR2 mcrB1 rpsL recA13*	26
JE177	R100-1/F$^-$ λ^- *mal-5*::*sfa-3 ton*	A. Nishimura

kanamycin (50 μg/ml), rifampin (100 μg/ml), chloramphenicol (30 μg/ml; Sigma), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 140 mM; Wako). The restriction enzymes *Bam*HI, *Eco*RI, *Xmn*I (New England Biolabs), *Kpn*I, *Pst*I, *Sph*I, and *Xho*I (Takara) and T4 DNA ligase (Takara) were used. PCR was carried out with AmpliTaq DNA polymerase (Perkin-Elmer Cetus). A kit, Muta-Gene (Bio-Rad), was used for mutagenesis. All the enzymes and kits were used as recommended by the suppliers.

Construction of pABO plasmids. To construct pABO401 (Fig. 1), plasmid pSI87-B19 was digested with *Pst*I and *Eco*RI, which cleave the pUC19 vector and R100 sequences, respectively, and the fragment containing the *Bam*HI-*Eco*RI segment of R100 corresponding to the region from bp −700 to 3020 was inserted into the multiple cloning site of plasmid pHSG299. To construct pABO411 (Fig. 1), pSI87-XE2 was digested with *Bam*HI and *Xmn*I, and the fragment corresponding to the region from bp −700 to 405 of R100 was inserted into pHSG299. To construct pABO421 (Fig. 1), pSI87-ES3 was digested with *Sph*I and *Xmn*I, which cleave the pUC19 and R100 sequences, respectively, and the fragment containing the *Eco*RV-*Xmn*I segment of R100 corresponding to the region from bp −246 to 405 was inserted into pHSG299. To construct pABO70, pABO701, pABO731, pABO741, pABO751, pABO761, and pABO771 (Fig. 1), the PCR-amplified fragments containing the relevant R100 segments shown in Fig. 1 were inserted into pHSG299. The synthetic oligonucleotide primers used for PCR are listed in Table 2.

To construct plasmids pABO422 to pABO437 (Fig. 2), pABO421 was first digested with *Sph*I and *Eco*RI, which cleave the pHSG299 vector sequence, and the fragment containing the *Eco*RV-*Xmn*I segment of R100 corresponding to the region from bp −246 to 405 was cloned into plasmid pUC119 to carry out site-directed mutagenesis of each specific site in the fragment. Then, after mutagenesis was performed according to the method of Kunkel (22) with the resulting pUC119 derivatives used as template and synthetic oligonucleotide primers (Table 2), the mutagenized plasmid was digested with *Sph*I and *Eco*RI and the fragment obtained was reinserted into pHSG299. The synthetic oligonucleotide primers used for mutagenesis are listed in Table 2.

To construct pABO821 (Fig. 1), pABO432 was digested with *Pst*I, which cleaves both the pHSG299 sequence and the mutated region in pABO432 (Fig. 2 and 3), and the *Pst*I fragment in which *oriT* is deleted was self ligated. To construct pABO831 (Fig. 1), a *Kpn*I restriction site was introduced by site-directed mutagenesis into pABO421 at the region of R100 from bp −21 to −16. Then, the resulting plasmid was digested with *Kpn*I, which cleaves both the pHSG299 sequence and the mutated region (Fig. 2 and 3), and the *Kpn*I fragment was inserted into pHSG299. To construct pABO841 (Fig. 1), pABO435 was digested with *Xho*I and *Eco*RI, which cleave the mutated regions in pABO435 and in the pHSG299 sequence, respectively, and the *Xho*I-*Eco*RI

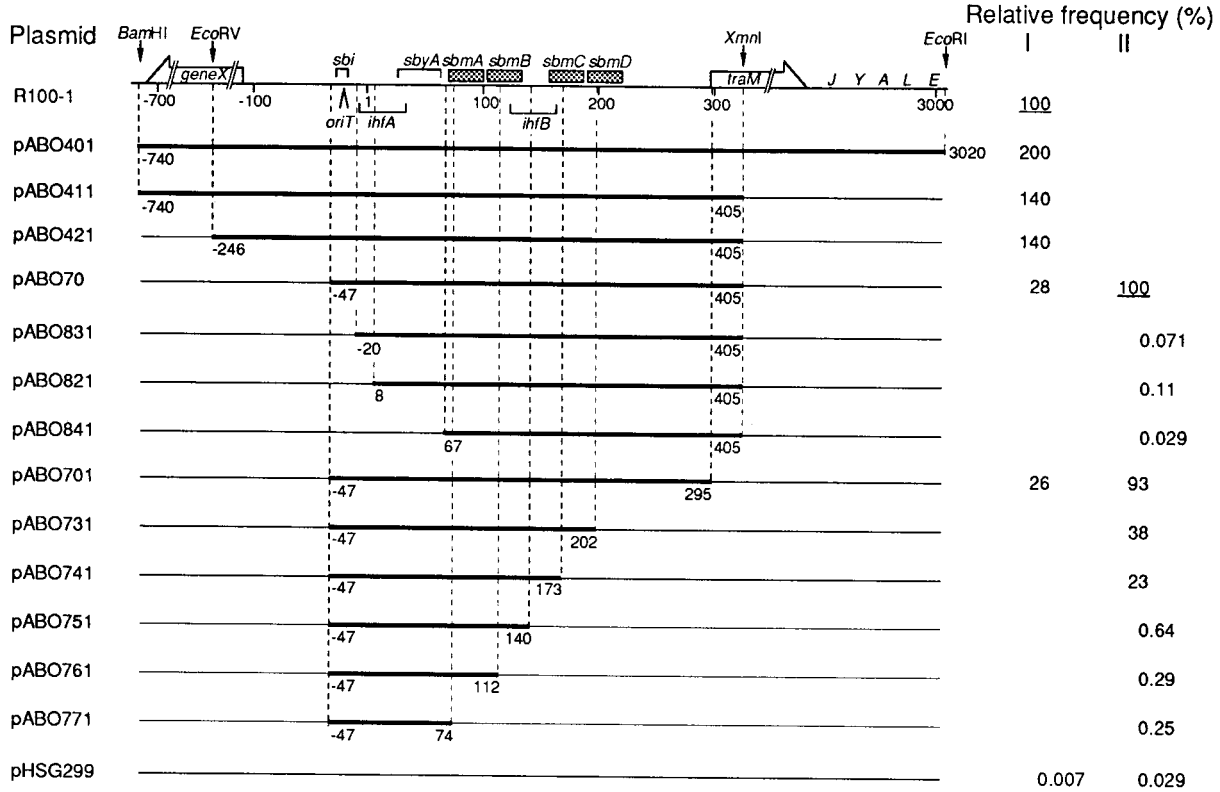

FIG. 1. pABO plasmids with or without deletions in the *oriT* region and relative frequencies of their transfer in the presence of R100-1. A map of the *oriT* region of R100 is shown at the top. Genes *X* and *traM* are delineated with thick open arrows. *J*, *Y*, *A*, *L*, and *E* indicate *tra* genes located downstream of *traM*. These genes are depicted in a small scale different from that of the other genes. The positions of four TraM-binding sites (*sbmA*, *sbmB*, *sbmC*, and *sbmD*) are indicated by shaded boxes. The TraI recognition site (*sbi*), IHF-binding sites (*ihfA* and *ihfB*), and TraY-binding site (*sbyA*) are marked by brackets. Numbers below the map indicate coordinates to the R100 sequence (Fig. 3) (2). Cleavage sites of relevant restriction enzymes used to clone the DNA segments (thick lines) in pABO plasmids are shown. Numbers in columns I and II are relative transfer frequencies, which were calculated by dividing the transfer frequency of a pABO plasmid by that of R100-1 or pABO70, respectively. Mating was carried out with *E. coli* NM554, harboring both R100-1 and a pABO plasmid, as the donor and DH1Rif as the recipient. Transconjugants harboring a pABO plasmid were obtained as Km^r Rif^r colonies, and transconjugants harboring R100-1 were obtained as Cm^r Rif^r colonies (see Materials and Methods). The data were obtained from 2 to 10 independent mating experiments (see Materials and Methods). The relative frequencies of transfer of pABO plasmids to R100-1 were calculated by dividing the number of Km^r transconjugants by the number of Cm^r transconjugants.

TABLE 2. Synthetic oligonucleotide primers used for PCR and site-directed mutagenesis

Sequence[a]	Position[b]	Plasmid constructed or site mutagenized[c]
5′-TACAAAATTACTggatCCATAAGATAAAAC-3′	311 to 282	pABO701
5′-CGTCCCTAATATAgATCtATGTTTTTCATG-3′	158 to 129	pABO751
5′-TATAAAAAAATGgGATCCGGCGCTAGGGGC-3′	187 to 158	pABO741
5′-AAAGACACTCCTAGggatcCCTCTAGGATCAT-3′	221 to 190	pABO731
5′-TGTCAGTACgGATCCTAATAA-3′	123 to 103	pABO761
5′-TTTCATggaTCCACCTCTGGTGAC-3′	−130 to −107	pABO421
5′-GTTggatCCACCAAAAGCACCACA-3′	−54 to −31	pABO70, -701, -731, -741, -751, -761, -771
5′-ACAGGATCcCCAACGACTCTCTAT-3′	83 to 60	pABO771
5′-ATCGCCAACGACTCTCTATgTtAacATTaAGctTTATatcgatTAAATAGCGTTTGTTAATTAC-3′	78 to 15	*sbyA*
5′-CCTAATAAGAGTCGCTAgctttgatagcagctATCGCCAACGACTCTCTA-3′	110 to 61	*sbmA*
5′-ATGTTTTTCATGAAAATTGagctgctatcaatgcATAAGAGTCGCTATAG-3′	140 to 91	*sbmB*
5′-AAATGCGATCCGGCGCgctgctatcaaagcATATATATCAATGTTTTTCA-3′	180 to 131	*sbmC*
5′-ACAAGTAAGAAAGACACTCagctgctatcaatccGGATCATCCTATAAAA-3′	230 to 181	*sbmD*
5′-TAAATAGCGTTTGcTgcagACggtaccTAAAACGTAAATC-3′	35 to −5	*ihfA*
5′-GTCCCTAATATATAgatcTtcTagaCATGAAAATTGTCAGTA-3′	157 to 118	*ihfB*
5′-CAGGATCGCCAACGACgaTCTCTATTTAATAATTC-3′	82 to 50	Site between *sbyA* and *sbmA*
5′-GGTCGTAACAGGATCGCCAACGACtcgagTCTCTATTTAATAATTCAGAATTATT-3′	90 to 41	Site between *sbyA* and *sbmA*
5′-GGTCGTAACAGGATCGCCAACGACtagtagaTCTCTATTTAATAATTCAGAATTATT-3′	90 to 41	Site between *sbyA* and *sbmA*
5′-GGTCGTAACAGGATCGCCAACGACtagtccatggTCTCTATTTAATAATTCAGAATTATT-3′	90 to 41	Site between *sbyA* and *sbmA*

[a] Lowercase letters in the nucleotide sequences indicate those nucleotides altered from the original sequences to introduce restriction sites useful for cloning or substitution or for insertion mutations at relevant sites.
[b] Numbers are coordinates given to the nucleotide sequence of R100 in Fig. 3.
[c] For specific sites mutagenized, see Fig. 3.

fragment was inserted into pHSG299. The nucleotide sequences of the critical regions of the plasmids constructed as described above were confirmed by the dideoxy-chain termination-sequencing method with the DEAZA-Sequencing kit (Takara).

Mating. The overnight culture of a donor strain, NM554, harboring both R100-1 and a pABO plasmid, was inoculated into 3 ml of fresh LB broth and incubated without shaking at 37°C for 3 h in the flask. The overnight culture of the recipient, DH1Rif, was also inoculated into fresh LB broth and then incubated with shaking at 37°C for 3 h. Then, 2.5 ml of this culture was added to the donor culture in the flask and incubated for mating for another 1 h at 37°C without shaking. After a 1-h incubation, the mating mixture was diluted and plated onto plates containing rifampin and kanamycin or chloramphenicol. Colonies formed after overnight incubation at 37°C were counted. The frequency of transfer of R100-1 was calculated by dividing the number of the Cm^r Rif^r transconjugants by the number of donor cells (Km^r Cm^r Lac^-). The frequency of transfer of the pABO plasmid was calculated by dividing the number of the Km^r Rif^r transconjugants by the number of donor cells. The data were obtained from 2 to 10 independent mating experiments. The standard deviations calculated were in the range of 6.9 to 97% of the average numbers obtained. The relative transfer frequencies were calculated as described in the legend to Fig. 1.

RESULTS

pABO plasmids with DNA segments in the *oriT* region and their mobilization by R100-1. pABO401 (Fig. 1) is a derivative of a kanamycin resistance plasmid, pHSG299, with the DNA segment of R100 corresponding to the region from bp −700 to 3020 (see Fig. 3 for the coordinate system given to the R100 sequence), which contains gene *X*, the *oriT* region, *traM*, *-J*, *-Y*, *-A*, *-L*, and a part of *traE*. pABO401 was transferred in the presence of R100-1, a transfer-proficient mutant of R100, to the recipient cells at a frequency (1.7×10^{-2} per donor cell) as high as that of R100-1 (8.0×10^{-3} per donor cell), while the parental plasmid pHSG299 was mobilized at a very low frequency (3.2×10^{-6} per donor cell) (see Fig. 1 for relative frequencies). Among the kanamycin-resistant transconjugants obtained from the mating experiment using the donor cell harboring pABO401 and R100-1, the majority harbored pABO401 alone (data not shown), showing that pABO401 was transferred independently to the recipient, not through the integration of pABO401 with R100-1. The Km^r transconjugants, however, which were obtained from the mating experiment using the donor cell harboring pHSG299 and R100-1, did not harbor pHSG299 but did harbor a large plasmid corresponding to R100-1 (data not shown), indicating that pHSG299 was cointegrated with R100-1 and transferred to the recipient cell. Probably, transposable DNA elements carried by R100-1 were involved in the cointegration of pHSG299 and R100-1.

We then examined other pHSG299 derivatives with a DNA segment containing the *oriT* region shorter than that in pABO401. Two plasmids, pABO411 and pABO421, were mobilized in the presence of R100-1 as frequently as was R100-1 itself (Fig. 1). This shows that the segment in pABO421 containing the region from the middle of gene *X* to the middle of *traM* includes the region required for DNA transfer. Two other plasmids, pABO70 and pABO701, containing all of the specific sites identified in the *oriT* region were mobilized at almost the same frequency, which is, however, slightly lower than that of pABO421 but much higher than that of pHSG299. This shows that the region between gene *X* and *sbi* in pABO421 is needed for efficient transfer but is not absolutely required for DNA transfer.

Effects of deletions in the *oriT* region on DNA transfer. To determine whether the specific sites previously identified in the *oriT* region in vitro are important for DNA transfer, various plasmids with deletions in the *oriT* region in pABO70 were constructed (Fig. 1) and tested for their mobilization in the presence of R100-1. In one series of plasmids from which the region on the left side of the *oriT* region was deleted, three plasmids, pABO831, pABO821, and pABO841, were not mobilized (Fig. 1); this indicated that *sbi*, which was deleted from each of these plasmids, is indispensable for DNA transfer. In the other series of plasmids from which the region on the right side of the *oriT* region was deleted, two plasmids, pABO731 (with *sbmD* deleted) and pABO741 (with *sbmC* and *sbmD* deleted), were mobilized at frequencies lower than that of pABO70 (Fig. 1). The other three plasmids, pABO751 (with *ihfB*, *sbmC*, and *sbmD* deleted), pABO761 (with the region

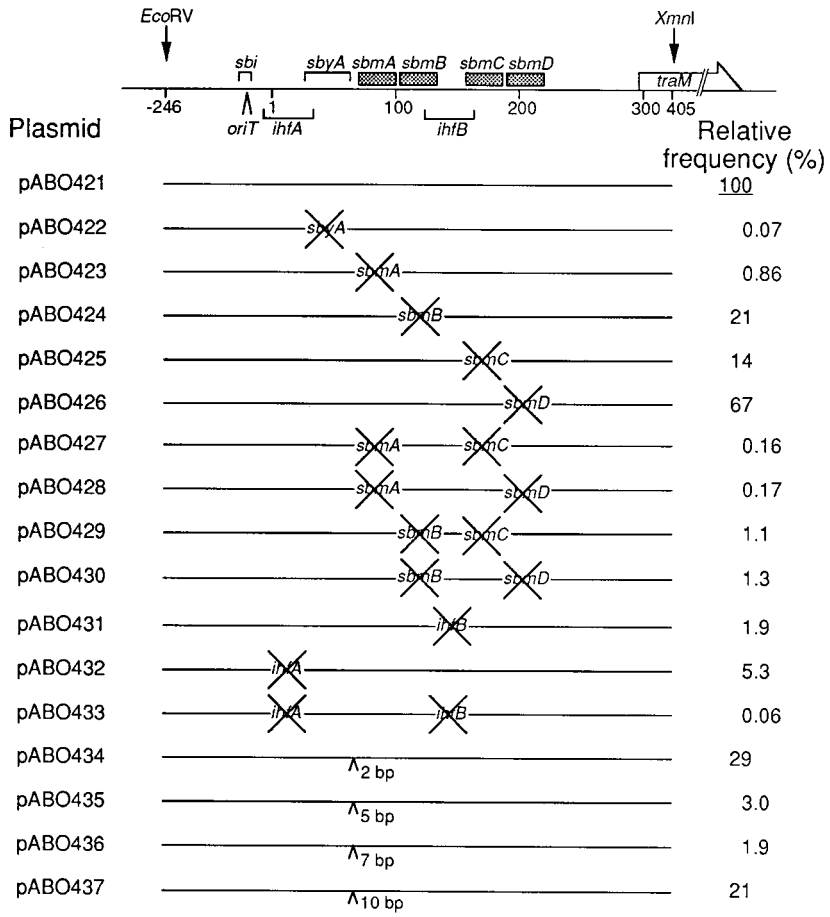

FIG. 2. pABO plasmids with substitutions or an insertion mutation and relative frequencies of their transfer. A map of the *oriT* region of R100 is shown at the top (Fig. 1). pABO plasmids were constructed by site-directed mutagenesis at the positions indicated (Fig. 3). pABO434 to pABO437 have insertions of 2, 5, 7, and 10 bp, respectively, between *sbyA* and *sbmA*. Numbers are frequencies of transfer relative to that of plasmid pABO421 and were calculated as described in the legend to Fig. 1 from the data obtained from 2 to 10 independent mating experiments (see Materials and Methods).

from *sbmB* to *sbmD* deleted), and pABO771 (with the region from *sbmA* to *sbmD* deleted), were transferred at frequencies lower than those of pABO731 and pABO741 (Fig. 1). These results indicate that all of the *sbm* sites and *ihfB* are not equally important but do contribute to the efficient transfer of plasmid DNA.

Effects of substitution mutations introduced into each specific site on DNA transfer. To determine whether each specific site in the *oriT* region is important for DNA transfer, substitution mutations were introduced by site-directed mutagenesis into each site on the DNA segment in plasmid pABO421 (Fig. 2) and the resulting pABO plasmids were tested for their mobilization in the presence of R100-1. Note that in this mutagenesis, substitutions were introduced to completely alter the consensus sequence previously determined for *sby* (18), *ihf* (10), and *sbm* (1) (Fig. 2 and 3). Plasmid pABO422, with substitutions in *sbyA*, was not mobilized (Fig. 2), showing that *sbyA* is essential for DNA transfer. Plasmids pABO423 to pABO426, with substitutions in *sbmA* to *sbmD*, respectively, were mobilized at frequencies lower than that of pABO421 (Fig. 2). Plasmids pABO427 to pABO430, with substitutions in two of four *sbm* sites, were mobilized at frequencies much lower than the expected frequencies, which can be calculated from the frequencies of transfer of plasmids with mutations in one site (Fig. 2). These results support the above-described indication that the four *sbm* sites are not equally important but contribute to efficient DNA transfer.

Two plasmids, pABO431, with substitutions in *ihfB*, and pABO432, with substitutions in *ihfA*, were mobilized at low frequencies (Fig. 2). pABO433, with substitutions in both *ihfA* and *ihfB*, was mobilized at a very low frequency (Fig. 2). These results show that both IHF-binding sites, *ihfA* and *ihfB*, in the *oriT* region are important for DNA transfer.

Effects of insertions in the region between *sbyA* and *sbmA* on DNA transfer. To determine whether there is any relation between the *sbi-ihfA-sbyA* region and the *sbm* region, we introduced several-base-pair insertions into the region between *sbyA* and *sbmA* and tested the mobilization of the resulting plasmids, pABO434 to pABO437 (Fig. 2), in the presence of R100-1. Plasmids pABO434 to pABO436, with insertions of 2, 5, and 7 bp, respectively, were mobilized at frequencies that declined as the lengths of the insertions increased (Fig. 2), but pABO437, with an insertion of 10 bp, was mobilized at a frequency higher than those of the others (Fig. 2). Considering the helical turn of B-DNA to be about 10.5 bp per turn, this result suggests that phasing between *sbyA* and *sbmA* is important for DNA transfer. We discuss below that two complexes are formed on both sides of the insertion and that an insertion of less than 10 bp disturbs the functional phase, causing inefficient DNA transfer.

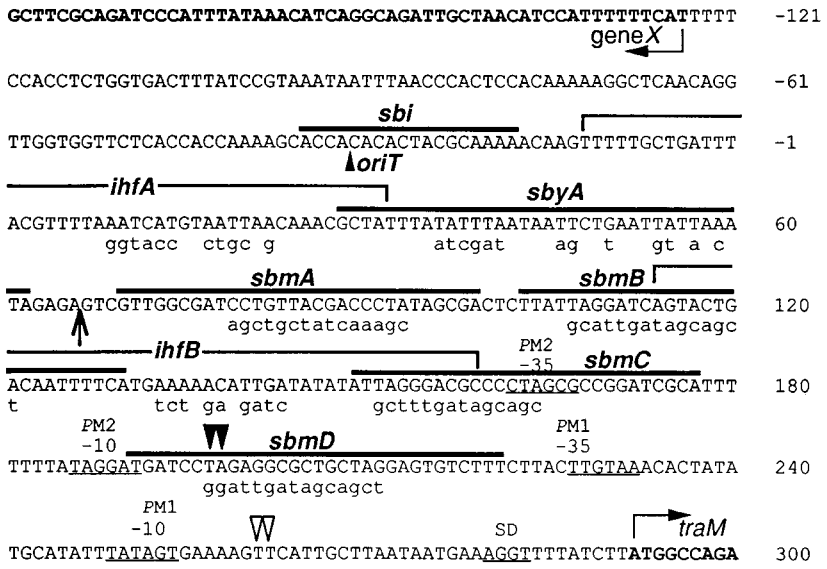

FIG. 3. Nucleotide sequence of the *oriT* region of R100. *oriT*, origin of DNA transfer (solid triangle); *sbi*, TraI recognition site (thick bar); *sbyA*, TraY-binding site (thick bar); *sbmA*, *-B*, *-C*, and *-D*, *TraM*-binding sites (thick bars); *ihfA* and *ihfB*, IHF-binding sites (brackets); SD, ribosome binding site for *traM* (underlined); P_{M1} and P_{M2}, promoters for *traM*. Transcription start sites in p_{M1} and p_{M2} are indicated by open and solid inverted triangles, respectively. Numbers indicate coordinates to the R100 sequence defined previously (2). Coding sequences of *traM* and gene *X* are shown in boldface type. Substitution mutations introduced into each specific site are shown in lowercase letters below the nucleotide sequence of each site. Site-directed mutagenesis was carried out with primers listed in Table 2 (Fig. 2). Note that substitutions introduced into *sbyA* altered the consensus sequence derived from three *sby* sites (18). Substitutions introduced into *sbm* sites altered the consensus sequence derived from four *sbm* sites (1). Substitutions introduced into *ihfA* and *ihfB* altered the consensus sequence of IHF-binding sites (10). The arrow between *sbyA* and *sbmA* indicates the position of insertions introduced with the primers listed in Table 2 (Fig. 2).

DISCUSSION

In this paper, we have demonstrated that the *sbi* site, which contains *oriT*, and its neighboring sites, *ihfA* and *sbyA*, are important for DNA transfer in vivo. This may support the previous proposal that TraI, IHF, and TraY, which recognize or bind to *sbi*, *ihfA*, and *sbyA*, respectively, form a specific complex (called *oriT*-some) at these sites to efficiently introduce the nick at *oriT* (17, 20). It is likely that IHF, known to activate various biological processes by bending DNA after binding to a specific site (10), is involved in the formation of *oriT*-some by binding to *ihfA*, which is located between *sbi* and *sbyA*.

In this paper, we have also demonstrated that the other sites located in the *oriT* region, namely, *sbm* sites and *ihfB*, are important for DNA transfer in vivo. This may support the previous proposal that TraM and IHF bind to *sbm* and *ihfB*, respectively, and form a functional complex (called the TraM-IHF complex) to accomplish a high frequency of DNA transfer (2). It is likely that IHF is also involved by binding to *ihfB*, located in the middle of the *sbm* region, in the formation of the TraM-IHF complex. We have previously reported that in the *sbm* region, *sbmC* and *sbmD* overlap one of the two promoters for the *traM* gene, p_{M2} (Fig. 3), and that the binding of TraM to these sites autoregulates the expression of *traM* (2). We assume that TraM, which binds to the two *sbm* sites, is involved not only in the autoregulation of the expression of its own gene but also in the formation of the TraM-IHF complex, which is active in DNA transfer. We have discussed previously that TraM may have the function of anchoring the *oriT* region to the membrane to facilitate DNA transfer (1). This function of TraM is likely to be accomplished through the formation of the TraM-IHF complex.

As described in Results, a deletion in the region between gene *X* and *sbi* slightly lowered the transfer frequency (Fig. 1), suggesting that there exists another functional site involved in efficient DNA transfer. Note that this is a proximal region transmitted to the recipient cell after the TraI protein introduces a nick at *oriT* and unwinds the double-stranded DNA from the nick. It is thus possible that the deletion in this region (called the leading region) has caused the reduction of the transfer frequency.

It has been previously reported that the transfer frequency of R100-1 in IHF^- cells is lowered to about 0.1 to 1% of that in IHF^+ cells (6, 14). Although the IHF^- mutation causes pleiotropic effects, this result suggests that IHF is involved in the transfer of R100-1. Our observation that substitutions introduced into *ihfA* or *ihfB* reduced transfer frequencies could directly show that IHF is actually involved in DNA transfer by binding to either one of the IHF-binding sites in the *oriT* region. We have also observed in this study that the mutant with substitutions in both *ihfA* and *ihfB* has lost its transfer ability almost completely. It is noteworthy that this effect is greater than that observed in the genetic experiment using the IHF^- mutant as a host. It is possible that there exists a host factor which can replace IHF in IHF^- cells, such as the histone-like protein HU, which is structurally and functionally similar to IHF (10, 21, 29), and suppress transfer ability to a certain extent. Considering our previous observation that IHF partially represses TraM expression (2), it is also possible that a greater amount of TraM, more of which is supposed to be produced in IHF^- cells than in IHF^+ cells, suppresses transfer ability in the absence of IHF.

In this paper, we have demonstrated that two regions that include *sbyA* and *sbmA* have to be located spatially in an appropriate position along the DNA helix to facilitate transfer. This suggests that there is some communication between *oriT*-some and the TraM-IHF complex. As described in Results, pABO423, with substitutions in *sbmA*, showed a transfer frequency lower than those of pABO424 to pABO426, with substitutions in *sbmB*, *sbmC*, and *sbmD*, respectively (Fig. 2). This

suggests that *sbmA* bound by TraM has an important role in communicating with *oriT*-some.

As mentioned earlier in this section, TraM is supposed to anchor the *oriT* region at the membrane. If this is the case, the position where the *oriT* region is anchored should be close to the membrane pore, through which DNA is transmitted. TraD, which is another protein required for DNA transfer, is believed to form the membrane pore, and thus it is likely that TraD and TraM in the TraM-IHF complex form a DNA transfer apparatus at the early stage of DNA transfer. It is also likely that TraI in *oriT*-some, which introduces the nick at *oriT*, interacts with TraD and that the TraM-IHF complex helps in this interaction. However, there has been no evidence suggesting an interaction between TraM and TraD or between TraI and TraD either in vivo or in vitro.

The *oriT* regions of sex factor plasmids F and R1, which are closely related to R100, are homologous to the *oriT* region of R100. The binding sites of TraY, TraM, and IHF in the *oriT* regions of F and R1 have been identified and found to be arranged similarly to those in the *oriT* region of R100 (7, 8, 30). This result and the results of deletion analysis of the *oriT* region of F (12, 15) suggest that there exists a common molecular mechanism in DNA transfer that is promoted by sex factors related to R100.

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