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Functional Relationship Between Parts of the Replication Region of Plasmid ColE1

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The inhibition of plasmid ColE1 replication caused by a deletion of the ColE1 plasmid replication origin has been previously reported (T. Hashimoto-Gotoh and J. Inselburg, J. Bacteriol. **139**:597-619). Evidence is presented showing that restoration of the deleted nucleotide sequence in the precise relationship it normally has to the rest of the replication region is essential for restoration of ColE1 replication capability to the deletion mutant.

A 436-base pair region of ColE1 plasmid DNA that contains the plasmid replication origin (7, 8) possesses enough information to sustain it as an autonomously replicating multicopy plasmid in *Escherichia coli* (7). That region, which extends from 16 base pairs to the right (+) of the ColE1 replication origin to 420 base pairs to its left (−), will be referred to as the *rep* region (7, 8). We previously isolated a chimeric plasmid, pHSG124 (Fig. 1) that is formed by ligating a derivative of ColE1 (pDMS630) and a temperature-sensitive replication derivative of pSC101 (pHSG1) (3). Under normal cell growth conditions the chimera exhibits ColE1-type replication (3). Deletions involving the ColE1 replication origin and adjacent nucleotide sequences in the ColE1 *rep* region of pHSG124 led to the deletion mutants being replicated by the pHSG1 component of the chimera. The mutant copy number is reduced from 20 to 4 per cell; the plasmid copy number cannot be amplified; and its replication becomes temperature sensitive (3). One such deletion mutant, pHSG210, was shown to have lost the replication origin and between 107 and 184 base pairs adjacent to it in the ColE1 *rep* region (Fig. 1) (3, 4). The block of the ColE1-directed replication process caused by the deletion in pHSG210 could be due to the deletion of information at or near the ColE1 replication origin utilized as a site or transcribed or to the disruption of an essential relationship that exists between the information in the deleted part of the *rep* region and the remainder of the *rep* region.

In the experiment reported here, I ask whether restoring the deleted *rep* region sequence of ColE1 DNA to any part of pHSG210 is sufficient to restore ColE1-mediated replication to that chimeric plasmid (Fig. 1a) or whether restored information must be restored in the same orientation and with the original continuity it had with the other portion of the ColE1 *rep* region (Fig. 1b or c).

The deleted *rep* region of pHSG210 is contained in a small *Hae*II endonuclease-generated fragment of ColE1, the *Hae*II E fragment (Fig. 1 and 2), and the remainder of the *rep* region is located at the end of an adjacent *Hae*II endonuclease-generated fragment, the *Hae*II A fragment (Fig. 1 and 2). The restoration of the deleted sequence could therefore be achieved by reintroducing the *Hae*II E fragment back into the pHSG210 plasmid. pHSG210 was partially digested with *Hae*II endonuclease to provide insertion sites for the *Hae*II E fragment, and ColE1 DNA was completely digested with *Hae*II endonuclease (Fig. 1) to provide both *Hae*II E and A fragments. The digested DNAs were mixed and ligated with T4 DNA ligase as previously described (3, 4), and ampicillin-tetracycline-resistant transformants of *E. coli* strain P678-54 (3, 4) that exhibited stability at high temperatures were selected. Those derivatives of pHSG210 had presumably regained the ColE1 replication ability. Plasmid DNAs isolated from 10 transformants were shown to have simultaneously conferred both drug resistances on cells and regained the ColE1-mediated replication characteristics of being temperature resistant and replicating in the presence of chloramphenicol (1). Their sizes were about the same as but usually distinguishable from pHSG210 as determined by agarose gel electrophoretic analysis (data not shown). Variations in the size of the newly formed plasmids were due to deletions caused by more than one *Hae*II-generated break in the partially digested molecules and by additions of fragments that we presume conferred the ColE1-type replication characteristic of the plasmid.

The plasmids that exhibited temperature-resistant replication were completely digested with

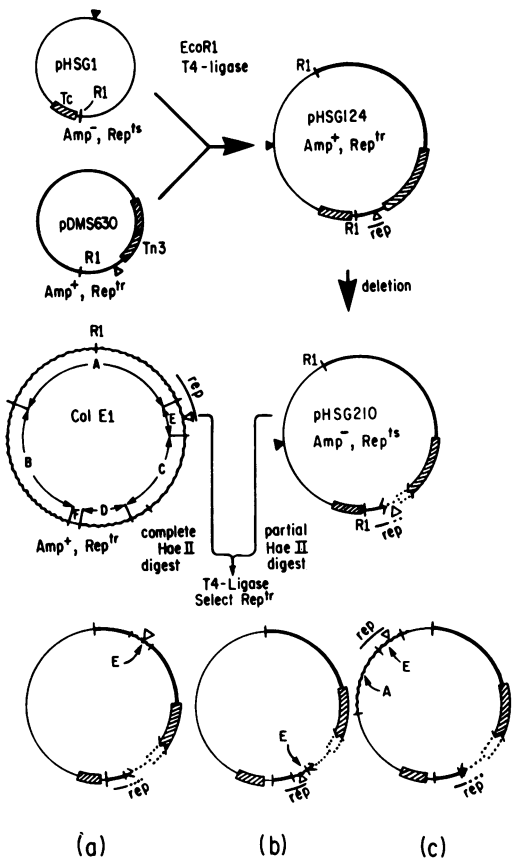

FIG. 1. *Diagrammatic representation of the origin of the chimeric plasmid pHSG210 and of the experiment that restores ColE1-mediated replication capacity back to it. pHSG1 is a temperature-sensitive, replication-defective mutation of pSC101 that carries genetic information that confers tetracycline resistance on cells (3). pDMS630 is a derivative of ColE1 containing a Tn3 transposon with the β-lactamase gene that confers ampicillin resistance on the cell carrying the transposon. pHSG124 is a chimera that exhibits the replication characteristics of ColE1. Amp⁺ and Amp⁻, the amplifiability of plasmid copy number in the presence of chloramphenicol (1, 3, 4); Rep^tr or Rep^ts, the temperature-resistant or temperature-sensitive nature of the plasmids replication, respectively. △ and ▲, the replication origins of ColE1 and pHSG1 (3), respectively. rep, the replication region of ColE1; (·····), the portion of pHSG124 deleted in forming pHSG210; (↓), the fused ends of the deletion; R1, the EcoRI endonuclease-sensitive site. A to F, HaeII endonuclease-generated fragments of ColE1 DNA (4, 6); (a) the insertion of the HaeII E fragment at some HaeII-sensitive site in pHSG210 other than that one that restores the complete rep region structure; (b) the insertion of only the HaeII E fragment to restore the complete rep region structure; (c) the insertion of both a HaeII A and E fragment to restore the complete rep region structure. Diagrams are not to scale.*

*Hae*II endonuclease, and the fragments produced were analyzed electrophoretically to determine whether the plasmids had acquired the *Hae*II E and or *Hae*II A fragment of ColE1 (Fig. 3). All the temperature-resistant plasmids isolated had, as predicted, acquired the *Hae*II E fragment (Fig. 3b–d), and some had acquired the *Hae*II A fragment as well (Fig. 3b and d). *Hae*II digests of pHSG210 contained neither a *Hae*II E fragment nor a *Hae*II fragment the size of the *Hae*II A fragment (2) (Fig. 3e). The presumptive *Hae*II A fragment in some plasmids was further identified by demonstrating that it contained an appropriately located *Eco*RI-sensitive site (Fig. 1; data not shown). The relationship of the newly inserted *Hae*II E fragments to either the remainder of the ColE1 *rep* region located in the *Hae*II A fragment of pHSG210 (Fig. 1b) or to another *Hae*II A fragment simultaneously ligated to pHSG210 (Fig. 1c) was examined by digesting the plasmids with either the *Taq*I or *Mbo*II endonuclease. These enzymes can generate specific ColE1 DNA fragments, *Mbo*B and *Taq*A (Fig. 2; Ohmori and Tomizaqa, personal communication) that span the *Hae*II A-E fragment junction. Those fragments are only formed if the *Hae*II A and E fragments are joined as in ColE1. The ends of each of those fragments are defined by an endonuclease-sensitive site within the region of the *Hae*II E fragment deleted from pHSG210 and another site in the *Hae*II A fragment (Fig. 2). It is seen that the specific *Taq*A and *Mbo*B fragments are not generated by digestion of pHSG210 (Fig. 3g and r). If the newly acquired *Hae*II E fragment of the temperature-resistant plasmids has been integrated in the same relation to the *Hae*II A fragment as it has in ColE1, then both the *Taq*A and *Mbo*B fragments should be recoverable. That is exactly what is found in endonuclease digests of all the plasmids isolated. In the selected digests shown in Fig. 3h–j and l, all the mutants contain a fragment the size of a *Taq*A fragment of ColE1 (Fig. 2) which is missing from pHSG210 (Fig. 3g). In the selected digests shown in Fig. 3m–p, all the mutants contain a fragment the size of an *Mbo*B fragment of ColE1 (Fig. 2) which is missing from pHSG210 (Fig. 3r). The test of the insertion location and orientation by the two enzymes makes an artifactual result caused by the coincidental generation of fragments the sizes of the *Mbo*B and *Taq*A fragments (Fig. 2) very improbable. The result supports the interpretation that restoration of the ColE1-mediated replication to the 10 temperature-resistant replication derivatives of pHSG210 was caused by the reestablishment of a complete ColE1 *rep* region in the plasmid and not the random inser

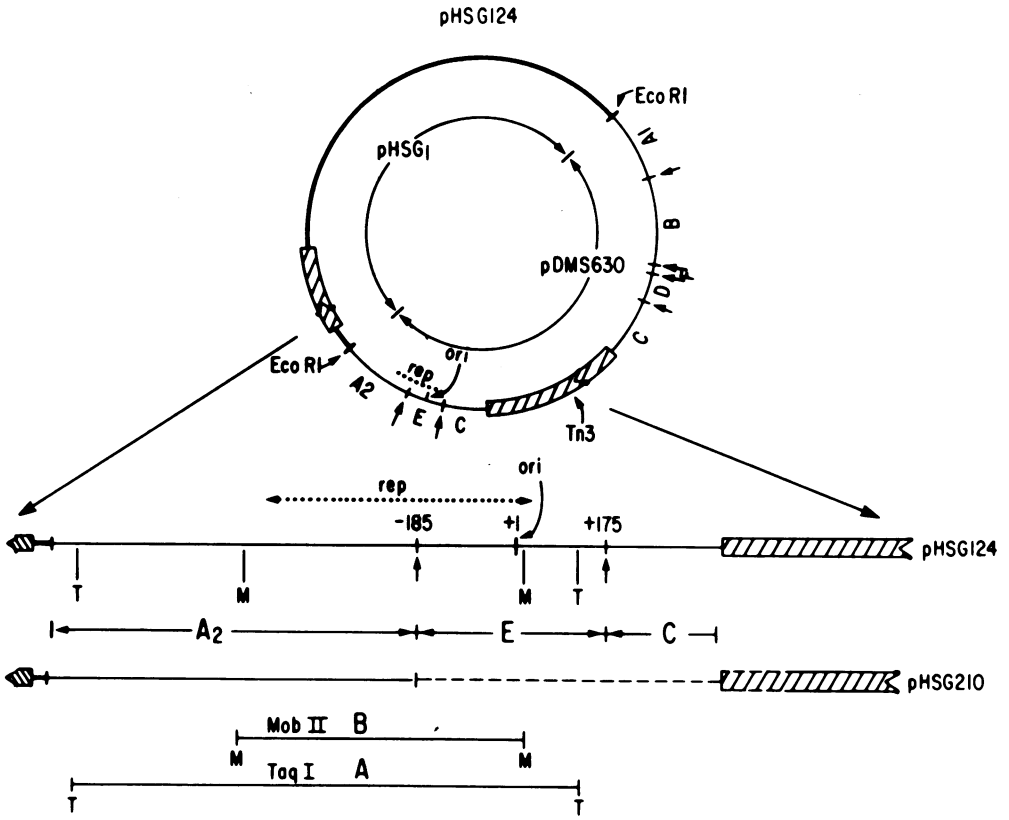

FIG. 2. *Diagrammatic representation of some endonuclease-sensitive sites in pHSG124 used to determine the site of insertion and the orientation of the ColE1 HaeII E fragment in pHSG210 derivatives. The location of endonuclease-sensitive sites shown are EcoRI, HaeII (↑), MboII (M) (Ohmori and Tomizawa, personal communication), and TaqI (T) (Ohmori and Tomizawa, personal communication). Only all the EcoRI-sensitive sites are shown. The HaeII A fragment of ColE1 (Fig. 1) is split by inserting a DNA segment into the ColE1 EcoRI site. The terms A1 and A2 represent those parts of the HaeII A fragment generated by its cleavage with EcoRI endonuclease (6, 8). The deleted portion of pHSG210 (·····) which terminates between base pair −107 and −184 in the HaeII E fragment (4) leaves the HaeII-sensitive site at base pair −185 intact but removes both the MboII- and TaqI-sensitive sites in the HaeII E fragment. The MboB fragment contains 663 base pairs, and the TaqA fragment contains ca. 1,145 base pairs (Ohmori and Tomizawa, personal communication). "ori" is the replication origin of ColE1 identified as base pair +1 (8).*

tion of the *Hae*II E fragment. In those plasmids that had only acquired the *Hae*II E fragment (e.g., Fig. 3c), that fragment must be inserted at the *Hae*II A-E fragment junction in the same orientation as that of the wild type (Fig. 1b). In those plasmids that had acquired both a *Hae*II A and E fragment (e.g., Fig. 3b and d), these data do not permit the distinction between the models "b" and "c" in Fig. 1, but the restoration of the wild-type relation of the *rep* region in the joined *Hae*IIA and E fragments must have occurred. The result therefore strongly supports the interpretation that the failure of pHSG210 to exhibit ColE1-mediated replication is not simply due to the deletion of the ColE1 replication origin or the loss of direct expression of information restricted to the *Hae*II E fragment of ColE1. The result suggests that expression of ColE1-mediated replication requires the maintenance of an essential specific relationship between the information defined by the deleted portion of the *rep* region in the *Hae*II E fragment and the part of the *rep* region in the *Hae*II A fragment. It can not be decided at this time if the essential specific relationship effects a transcriptional process crossing the *Hae*II-sensitive site at the *Hae*II A-E fragment junction or a structural relationship that, for instance, effects the expression of the ColE1 replication origin. As a number of reports indicate that a ColE1 plasmid polypeptide is not essential for ColE1-mediated plasmid replication (2, 5), the latter

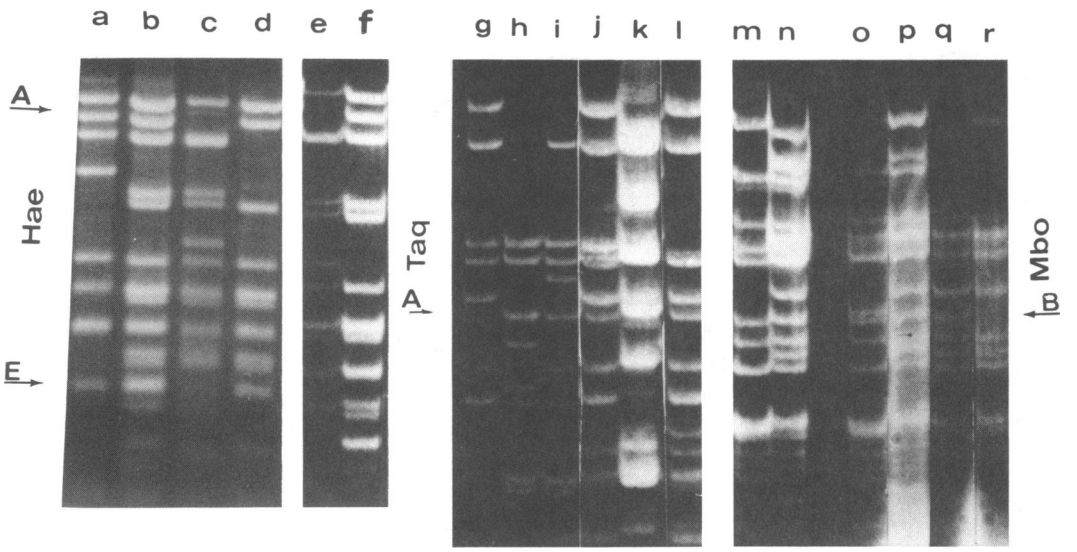

FIG. 3. *Agarose gel electrophoretic analysis of HaeII, TaqI, and MboII endonuclease-digested DNAs that were isolated from temperature-resistant replication derivatives of pHSH210. DNA of pHSG210 and representative temperature-resistant replication derivatives described in the text (pDMS1012, pDMS1013, pDMS1014, pDMS1016, pDMS1017), as well as control DNA of pDMS630 and pHSG124, were treated with either HaeII, TaqI, or MboII endonuclease (New England Biolabs). HaeII digestion reaction conditions were reported previously (3). TaqI digestion reactions containing 6 mM Tris-hydrochloride (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100 μg of sterile (autoclaved) gelatin per ml, enzyme, and DNA are done at 65°C. MobII digestion reactions containing 6 mM KCl, 10 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 μg of bovine serum albumin per ml, enzyme, and DNA are done at 37°C. Fragments were analyzed by agarose gel electrophoresis using a 1.4% agarose gel as previously described (3). Data relating to some of the temperature-resistant replication mutants are shown. (a–f) The HaeII endonuclease digests of plasmid DNAs: (a) pDMS630; (b) pDMS1013; (c) pDMS1017; (d) pDMS1014; (e) pHSG210; (f) pDMS1013. The location of the HaeII A and E fragments of ColE1 are indicated to the left of the lanes. (g–l) The TaqI endonuclease digests of plasmid DNAs. (g) pHSG210; (h) pDMS1014; (i) pDMS1013; (j) pDMS1016; (k) pDMS630; and (l) pDMS1017. The position of the Taq A fragment which is missing in (g) and present in all other lanes is indicated to the left of the lanes. (m–r) show the MboII endonuclease digests of plasmid DNAs: (m) pDMS1014; (n) pDMS1012; (o) pDMS1013; (p) pDMS1016; (q) pHSG124; (r) pHSG210. The position of the MboII B fragment which is missing in (r) and present in all other lanes is shown to the right of the lanes.*

possibility presently appears to be the more interesting interpretation.

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