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Recombination Sites in Plasmid Drug Resistance Gene Amplification

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The resistance plasmid NR1 derivative pRR330 consists of a neomycin-kanamycin resistance gene (*neo-kan*) flanked by directly repeated sequences of both insertion element IS*1* DNA (768 base pairs) and 840 base pairs of DNA which are a part of the chloramphenicol acetyltransferase (*cam*) gene. Most *Escherichia coli* cell populations that were cultured in high neomycin concentrations carried plasmids whose *neo-kan* gene amplification was mediated either by IS*1* DNA or by *cam* DNA as homologous recombination sites. This suggests that the final amplified cell populations were the descendants of a single cell.

Selective amplification of antibiotic resistance genes has been observed in procaryotic (2, 4, 6, 8, 12, 14, 17, 18) and eucaryotic (15) systems. The bacterial R plasmid NR1 is one of the most extensively characterized systems of antibiotic resistance gene amplification (14). NR1 is 90 kilobase pairs (kbp) in size and consists of a resistance transfer factor which carries genes for plasmid replication and conjugal transfer and an r-determinants component which carries the majority of the resistance genes (13, 14). The selective amplification of NR1 drug resistance genes is mediated by recombination between directly repeated single copies of the insertion element IS*1* which flank the r-determinants component (14). Even though insertion and transposable elements are known to facilitate recombination and other genetic rearrangements (10), we have previously shown that a part of the chloramphenicol resistance gene (designated Δ*cam*) of a size comparable to IS*1* can also serve as a recombination site in DNA amplification of NR1 derivatives (11). Here we show that the Δ*cam* gene homology which is clearly not an insertion element is used as efficiently as IS*1* to generate tandem duplications when both types of homologous sequences are present on the same plasmid. Our experiments also suggest that an amplified cell population usually results from the growth of the descendants of a single cell carrying a spontaneously amplified plasmid.

To examine whether DNA sequences which do not contain insertion elements can be used as efficiently as IS*1*s in drug resistance gene amplification, the plasmid pRR330 was constructed (Fig. 1). pRR218 was derived from NR1 by deleting most of the r-determinants component to form a plasmid containing the *cam* gene on a 1.3-kbp segment which is flanked by direct repeats of IS*1* (14). pRR720 was derived from pRR218 by subcloning by *Pst*I partial digestion (Fig. 1). The remaining 28 kbp of NR1 DNA on pRR720 contains the plasmid replication genes, the *cam* gene flanked by direct repeats of IS*1*, and the *stb* locus which mediates stable plasmid inheritance (13). The ampicillin resistance transposon Tn*3* was inserted into the remaining resistance transfer factor region of pRR720 near $IS1_b$ to form pRR315. The mini-F plasmid pML31 (7) consists of *Eco*RI fragment 5 of the F plasmid ligated to an *Eco*RI fragment containing a functional neomycin-kanamycin resistance gene (*neo-kan*) and a partial *cam* gene. This *neo-kan* fragment was derived from the plasmid R6-5, which is closely related to NR1 (16). The *cam* DNA present on the *neo-kan* fragment is indistinguishable from the corresponding region of the NR1 *cam* gene. Insertion of this *neo-kan* fragment from pML31 into the *Eco*RI site within the *cam* gene of pRR315 in the proper orientation produced the plasmid pRR330 which confers resistance to neomycin-kanamycin, chloramphenicol, and ampicillin (Fig. 1). pRR330 carries the *neo-kan* gene flanked by directly repeated single copies of both IS*1* (768 base pairs) and approximately 840 base pairs of a homologous sequence which is part of the *cam* gene (designated Δ*cam* in Fig. 1) and therefore unlikely to be an insertion element.

Selection for plasmid drug resistance gene amplification was carried out by subculturing *Escherichia coli* KP245 (6, 9) containing pRR330 in Penassay broth (Difco Laboratories, Detroit, Mich.) containing 200 μg of neomycin per ml. Stationary-phase cultures were diluted 10^{-8}-fold into medium with or without neomycin and grown to stationary phase 3 to 4 times in succession. Appropriate dilutions of cultures were spread onto plates containing either no drugs or various concentrations of neomycin, kanamycin, or ampicillin. Cells cultured in medium containing no antibiotic had a plating efficiency of approximately 10^{-5} on plates containing 200 μg of neomycin per ml. Cells cultured in medium containing 200 μg of neomycin per ml had a plating efficiency of approximately 1.0 under these conditions. Kanamycin resistance was also increased to a similar extent when cells were previously cultured in medium containing 200 μg of neomycin per ml. Resistance to ampicillin remained unchanged, indicating that the increase in neomycin and kanamycin resistance observed was not caused by an increase in plasmid copy number.

The selective simultaneous increase in neomycin and kanamycin resistance could result from a mutation in the resistance gene or its promoter or from selective amplification of DNA carrying the *neo-kan* resistance gene. These possibilities can be distinguished by restriction endonuclease analysis of pRR330 DNA from cultures with increased drug resistance. Restriction digests of a plasmid with a mutated gene or promoter would have the same fragment patterns as pRR330 (Fig. 2A). Selective amplification of the *neo-kan* gene, which is mediated by the flanking IS*1* elements or by the Δ*cam* DNA regions, will result in the formation of repeated, tandem sequences whose size will depend on which region of homology is used as the recombination site. The size and amount of amplification of the repeated se-

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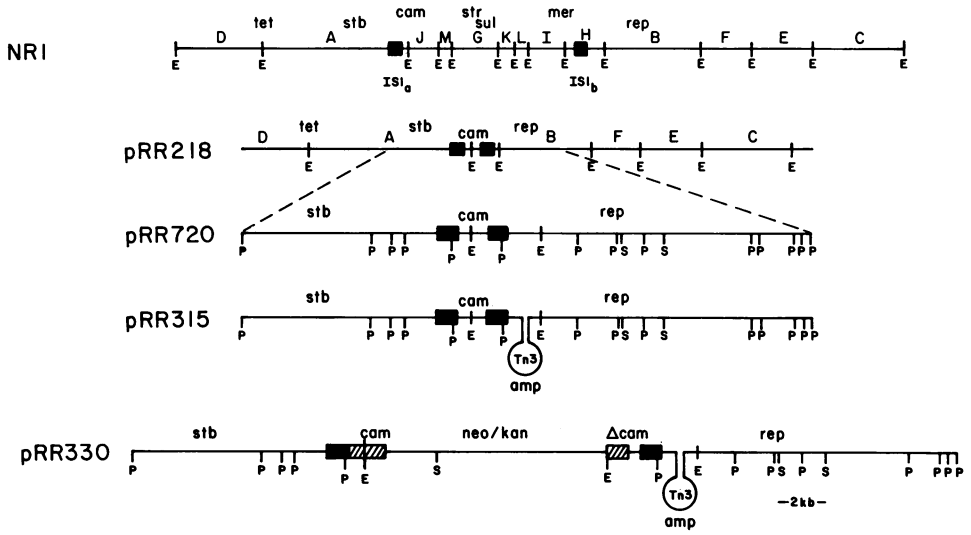

FIG. 1. Derivation of pRR330. The letters (A through M) on the *Eco*RI restriction map of NRI designate the thirteen largest *Eco*RI fragments of the plasmid DNA in order of decreasing size. pRR218 was derived from NR1 by deletion of most of the r-determinants DNA. pRR720 was then derived from pRR218 by partial *Pst*I digestion. Tn*3* was inserted by transposition to form pRR315. Finally, the *neo-kan* fragment at pML31 was inserted into pRR315 to form pRR330. Restriction endonuclease sites are indicated by letters below the linearized plasmid maps: E, *Eco*RI; P, *Pst*I; S, *Sal*I. *Pst*I and *Sal*I sites are not indicated on NR1 or pRR218. Symbols: ■, IS*1*; ▨, *cam* or Δ*cam* DNA (see text).

quences can readily be determined by cleavage with a restriction endonuclease such as *Sal*I, which has only a single cleavage site in the amplified segment. Recombination between *cam* DNA sequences would generate a 6.8-kbp fragment (Fig. 2B), whereas recombination between IS*1* elements would generate an 8.9-kbp fragment (Fig. 2C).

Plasmid DNA isolated from 15 independent cultures grown in medium containing 200 μg of neomycin per ml was digested with the restriction endonuclease *Sal*I, which has a single cleavage site within the *neo-kan* fragment. Representative restriction patterns are shown in Fig. 3. Of the amplified DNA samples, five had an 8.9-kbp fragment (e.g. lane 3), nine had a 6.8-kbp fragment (e.g. lane 4), and one had both novel fragments (lane 5). These results indicate that the *cam* DNA homology was used as efficiently as the IS*1* homology to generate duplication of the *neo-kan* gene. These results are also consistent with the presence of multiple autonomous *neo-kan* determinants in highly resistant cells. However, electrophoresis of undigested plasmid DNAs from such cells showed only large plasmids. DNA bands having the mobility expected for autonomous *neo-kan* determinants were not observed, suggesting that amplification was mediated by tandem duplication of the *neo-kan* determinants. Analysis of purified clones from the culture with both novel fragments showed that the culture was a mixture of cells containing one or the other type of amplified plasmids (data not shown). Both recombination sites were not used on a

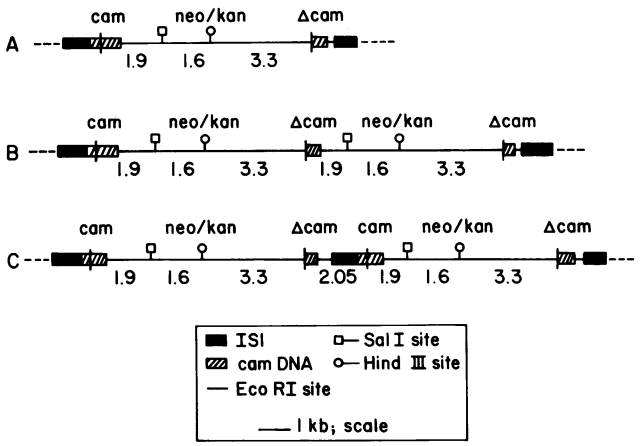

FIG. 2. Restriction endonuclease map of pRR330 and amplified pRR330. Only the resistance determinant parts of the plasmids are shown. (A) Unamplified pRR330. (B) pRR330 amplified with *cam* DNA sequence homology for initial recombination. (C) pRR330 amplified with IS*1* homology for initial recombination. Only two tandem r-determinants are shown on each plasmid. Numbers refer to the size (in kilobase pairs [kb]) of the DNA fragments between the restriction sites indicated.

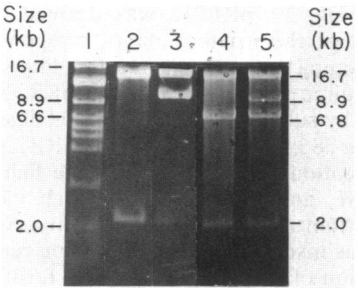

FIG. 3. Restriction endonuclease digestion of amplified pRR330. Plasmid DNA was isolated from cells cultured in Penassay broth (unamplified DNA) or in broth containing 200 μg of neomycin per ml (amplified DNA) and digested with *Sal*I. The top *Sal*I fragment in all pRR330 lanes is actually a doublet that can be resolved with further electrophoresis (data not shown). Lanes: 1, NR1 digested with *Eco*RI as size standards; 2, unamplified pRR330; 3 to 5, amplified pRR330. Lane 3 shows an 8.9-kbp fusion fragment, lane 4 shows a 6.8-kbp fusion fragment, and lane 5 shows both.

single plasmid molecule. The fact that only one of the two types of homologous sequences was used as a recombination site for DNA amplification in 14 of 15 cultures suggests that in the majority of instances amplified cultures resulted from the growth of descendants of a single cell. If descendants of more than one cell were present in the culture, both amplified fragments should have been present at a much higher frequency than was observed.

The R plasmid NR1 undergoes spontaneous amplification of its r-determinants component at a frequency of about 10^{-4} even in the absence of selection for increased drug resistance (4). In our present experiments strain KP245(pRR330) cells cultured in medium containing no antibiotic had a plating efficiency of approximately 10^{-5} on plates containing 200 μg of neomycin per ml. This suggests that pRR330 undergoes spontaneous amplification of the *neo-kan* resistance gene at a frequency of about 10^{-5} by using either IS*1* DNA or Δ*cam* DNA homologous sequences even in the absence of selection. Late exponential and stationary-phase cultures (1×10^9 to 5×10^9 cells per ml) of strain KP245(pRR330) would therefore contain approximately 10^4 cells per ml containing spontaneously amplified plasmids. Since stationary-phase cultures were diluted 10^{-8}-fold each time into Penassay broth containing 200 μg of neomycin per ml, it is unlikely that the initial cultures resulting from the first dilution contained cells with spontaneously amplified plasmids. It would appear that the cells in these initial cultures grew slowly or not at all until spontaneous amplification occurred in a single cell with either IS*1* DNA or *cam* DNA homology. The descendants of this cell would grow more rapidly owing to their increased drug resistance. The spontaneously amplified plasmids would also be able to undergo further amplification to produce cell cultures containing plasmids whose amplification was mediated only by either IS*1* DNA or *cam* DNA homology. In one culture both IS*1* DNA and *cam* DNA homology was found to mediate pRR330 amplification, although both recombination sites were not used on a single plasmid molecule. Our results, however, are inconsistent with the view that amplification is usually induced in a large number of cells directly by drug selection because this would have resulted in amplification mediated by both IS*1* elements and *cam* gene homology in all of the cultures.

Many of these questions have recently been discussed in relation to drug resistance gene amplification in normal and malignant mammalian cells (3). It is presently not known what determines the size of the amplified region and the frequency and amount of amplification in different mammalian cell lines. Our results imply that any region of sequence homology may be used to generate tandem duplications and their dissociation to form extrachromosomal elements which can be lost on prolonged growth in the absence of selection. It seems likely that the size of the amplified unit is determined by the distance between directly repeated, homologous chromosomal sequences which could be used as recombination sites. Since mammalian chromosomes contain many repeated sequences (1), there is considerable potential for spontaneous amplification of segments whose size and frequency of formation could be variable in different cell lines. Just as in the case of bacterial cells, mammalian cells with spontaneously amplified sequences would grow faster and undergo further amplification to produce the final cell population.

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