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Transformation of *Haemophilus influenzae* by Plasmid RSF0885 Containing a Cloned Segment of Chromosomal Deoxyribonucleic Acid

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A plasmid containing a single cloned insertion of *Haemophilus influenzae* chromosomal deoxyribonucleic acid that carried a novobiocin resistance marker was 2.6 times larger than the parent plasmid, RSF0885, which conferred ampicillin resistance. The most frequent type of transformation by this plasmid (designated pNov1) was the transfer of novobiocin resistance to the chromosome, with the loss of the plasmid from the recipient. In accord with this observation, after radioactively labeled pNov1 entered a competent cell, it lost acid-insoluble counts, as well as biological activity. The level of ampicillin transformation, which involved establishment of the plasmid, was almost two orders of magnitude lower than the level of novobiocin transformation. Both types of transformation were depressed profoundly in *rec-1* and *rec-2* mutants. Ampicillin transformants of wild-type cells always contained plasmids that were the same size as pNov1, although most of these transformants were not novobiocin resistant. Plasmid pNov1 in wild-type cells but not in *rec-1* or *rec-2* cells often recombined with the chromosome, causing a homologous region of the chromosome to be substituted for part of the plasmid, as shown by restriction and genetic analyses. Our data suggested that plasmid-chromosome recombination took place only around the time when the plasmid entered a cell, rather than after it became established.

Originally, cloning of *Haemophilus influenzae* DNA with an *H. influenzae* plasmid vector was undertaken in our laboratory in an attempt to analyze the products of various chromosomal loci where we isolated mutants defective in a number of aspects of genetic recombination and DNA repair. We have been sidetracked from this goal completely because of some unexpected phenomena which we observed in transformation by plasmids containing cloned fragments. The most important of these are (i) that one type of transformation involves integration of some of the cloned chromosomal DNA into the recipient chromosome, with a loss of the plasmid and the original plasmid antibiotic resistance marker, and (ii) that another type of transformation, in which a plasmid becomes established in the recipient cell and is the same size as the transforming plasmid, usually has the cloned DNA part of the plasmid altered. Thus, in most of the transformations where there is an addition of a plasmid to a recipient cell, the genetic marker of the cloned fragment in the incoming plasmid is lost and apparently is replaced by a segment of chromosomal DNA. In this study we investigated these phenomena.

MATERIALS AND METHODS

Microorganisms, transformation, gel electrophoresis, plasmid preparation, and electron microscopy methods are described in the accompanying paper (9).

Construction of plasmids containing cloned insertions. Purified RSF0855 and *H. influenzae* DNA containing eight antibiotic resistance markers were digested to completion with the restriction endonuclease *Pvu*II, as described in the accompanying paper (9). Ligation was performed for 2 h at 14°C in a reaction mixture (0.02 ml) containing 1 U of T4 ligase (New England Biolabs), 5 μg of plasmid DNA per ml, 1 μg of chromosomal DNA per ml, 66 mM Tris-hydrochloride (pH 7.5), 6.6 mM $MgCl_2$, 66 μM ATP, 10 mM dithiothreitol, and 20 mM spermidine (Sigma Chemical Co.).

Construction of library and isolation of cells containing particular genes. Competent strain BC200 cells were exposed to 0.03 μg of ligated DNA per ml, resulting in 9.5×10^3 transformants to ampicillin per ml. (Transformants were measured by plating the cells after exposure, incubating them for 90 min at 37°C, and then layering the plates with agar containing 10 μg of ampicillin per ml.) Selection was made by overnight growth in a liquid culture containing 1 μg of ampicillin per ml after expression in liquid growth medium, as described previously (11). Each ampicillin-resistant culture was stored frozen in 14% glycerol for future study. Samples were plated with

various antibiotics to isolate cells that were resistant to ampicillin and also contained a chromosomal marker. One of the larger plasmids, designated pNov1, was selected by plating with 2.5 μg of novobiocin per ml.

Measuring plasmid lengths. Electron microscope images on negatives were magnified 10-fold on the screen of a Nikon profile projector. Tracings were made, and the plasmid lengths were determined with a Numonics electronic digitizer.

Restriction endonuclease analysis. *Pvu*II digestion was as described in the accompanying paper (9). *Taq*I (New England Biolabs) digestion was carried out at 65°C for 1 h in a 0.05-ml reaction mixture containing 10 mM Tris-hydrochloride (pH 8), 6 mM $MgCl_2$, 100 mM NaCl, 6 mM beta-mercaptoethanol, 4 to 6 μg of plasmid DNA, and 7 U of enzyme.

Curing. Curing plasmid-bearing strains of their plasmids was done in liquid cultures containing 17 μg of acridine orange per ml, except for the Rec^- strains, where only one-half this concentration was used. Cells were grown for approximately six generations with acridine orange and then plated onto the surfaces of agar plates. The next day colonies were transferred with toothpicks to plates containing ampicillin or no drug. Each colony was also tested for novobiocin resistance.

Radioactively labeled plasmid. Cells were grown with aeration in 8 ml of brain heart infusion medium containing 10 μg of hemin per ml and 3 μg of NAD per ml to a density of approximately 10^8 cells per ml. Then 33 μCi of [^{3}H]thymidine (52 Ci/mmol) per ml was added, and growth was continued to saturation (under these conditions the specific activity of both plasmid DNA and chromosomal DNA was approximately 10^5 cpm/μg). A crude plasmid preparation was then made (9). This preparation was centrifuged at 32,000 rpm for 2 h in a 5 to 20% sucrose gradient containing 0.15 M NaCl and 0.015 M trisodium citrate in a Beckman SW50.1 rotor, assayed for radioactivity and transforming activity, and, in some cases, run on a gel. Under these conditions the residual chromosomal DNA in the plasmid preparation sedimented separately and faster than the plasmid DNA (data not shown). To investigate the fate of plasmid DNA during transformation, 12 ml of competent cells was exposed to 0.2 ml of the peak plasmid fraction from the sucrose gradient for 10 min at 37°C; then 20 μg of DNase per ml was added, and after a further incubation for 3 min at 37°C the mixture was centrifuged and resuspended at the same concentration in fresh MIV (12). Samples (4 ml) were withdrawn 0, 30, and 60 min later, and a crude plasmid preparation was made from each sample (9). The samples were then centrifuged for 2.25 h at 33,000 rpm. Approximately 12 fractions were collected from each sample in test tubes; a portion of each fraction was spotted onto paper and processed as previously described (3), and a portion was assayed for transforming activity.

RESULTS

Electron microscope determinations. Table 1 shows the electron microscopic measurements for plasmids pNov1 and RSF0885, compared with a plasmid pBR322 standard, since the size of the latter is known very precisely from a base sequence determination (13). The molecular weight of plasmid RSF0885 from these data was 3.7×10^6. Previous determinations of the molecular weight of this plasmid were 3.6×10^6 from gel data and 3.2×10^6 from electron microscopy without a standard (7). Another value from sedimentation velocity, gel electrophoresis, and electron microscopy was 4.1×10^6 (10). The molecular weight of pNov1 was 9.6×10^6 (Table 1).

Restriction analyses of the plasmids. The results of *Pvu*II digestions of pNov1 and RSF0885 are shown in Fig. 1. This restriction endonuclease made a single cut in RSF0885, resulting in a single band. In both cases before digestion there were two bands, which were from the relaxed forms and the supercoiled forms of the plasmids. After being cut by *Pvu*II, the larger plasmid (plasmid pNov1) showed two bands, one of which appeared to be identical to the single band of enzyme-treated RSF0885. The second band of pNov1 migrated more slowly than the first, indicating that this DNA was larger. These data showed that pNov1 contained only a single insertion of chromosomal DNA. The relative size of the chromosomal portion was consistent with the data in Table 1, in that the insertion in pNov1 was larger than the RSF0885 portion.

Figure 2 shows the results of digestions of pNov1 and RSF0885 with *Taq*I. The digested original plasmid (plasmid RSF0885) showed seven bands. With pNov1 six of these bands were present, and there were five additional bands.

DNA concentration dependence of transformation by pNov1. Figure 3 shows the dose-response curves for purified pNov1. The level of transformation to novobiocin resistance from pNov1 was almost two orders of magnitude higher than the level of transformation to ampicillin resistance from this plasmid. The level of novobiocin transformation was more than

TABLE 1. *Electron microscope measurements of RSF0885 and pNov1, using pBR322 as a standard*

Plasmid	No. of molecules measured	Magnification ($\times 10^3$)	Measured length (cm)	No. of base pairs	Ratio of length to RSF0885 length
pBR322	11	219	26.8 ± 1.6[a]	4,362	0.77
RSF0885	18	219	35.0 ± 1.0	5,700	1.0
pBR322	19	270	33.3 ± 1.1		
pNov1	10	270	115.7 ± 3.1	15,100	2.6

[a] Mean ± standard deviation.

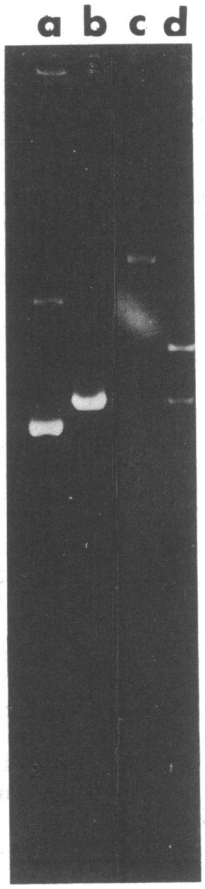

FIG. 1. *Gel electrophoresis of two plasmids before and after digestion with restriction endonuclease PvuII. Lane a, RSF0885; lane b, digested RSF0885; lane c, pNov1; lane d, digested pNov1.*

four orders of magnitude higher than the level of transformation for any marker from residual chromosomal DNA in preparations that were centrifuged twice through cesium chloride-ethidium bromide (data not shown).

Rec gene dependence of transformation by pNov1. Transformation by RSF0885 is not affected by the *rec-1* and *rec-2* mutations (9), but this was not the case for pNov1. The ampicillin transformation efficiency was considerably lower for the Rec$^-$ strains than for the wild type. A crude preparation of pNov1 produced 2.5×10^4 ampicillin transformants per ml with wild-type strain Rd, whereas it produced 25 and 60 ampicillin transformants per ml with *rec-1* and *rec-2* mutants, respectively. Transformation of the Rec$^-$ strains to novobiocin resistance by the plasmid was not observed under the conditions used. Transformation by chromosomal DNA is also very low in these strains (11).

Effect of *Pvu*II digestion on transformation by pNov1. *Pvu*II treatment resulted in a complete loss of the ability to transform to ampicillin resistance; undigested pNov1 from the sample shown in Fig. 1 produced 1.0×10^5 ampicillin transformants per ml, whereas digested pNov1 produced $<10^2$ ampicillin transformants per ml. However, transformation to novobiocin resistance was increased by *Pvu*II treatment. Undigested pNov1 produced 4.8×10^6 novobiocin transformants per ml, whereas digested pNov1 produced 3.1×10^7 novobiocin transformants per ml; this suggested that the nonhomologous part of plasmid pNov1 (from the parent, RSF0885) interfered with transformation when it was attached to chromosomal DNA.

Nature of the transformants from pNov1. To obtain information on the nature of the transformation that took place when competent cells were exposed to plasmid DNA containing an insertion, we isolated three different kinds of transformants: ampicillin transformants, trans-

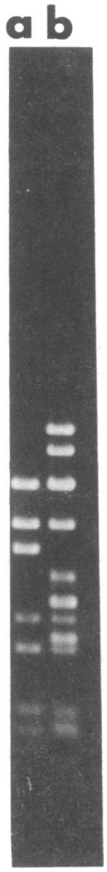

FIG. 2. *Gel electrophoresis of plasmids after digestion with restriction endonuclease TaqI. Lane a, RSF0885; lane b, pNov1.*

formants expressing only the inserted chromosomal marker, and double transformants from both of these. The following determinations were made for these transformants: whether they contained plasmids, the sizes of the plasmids, whether the cells were resistant to the unselected marker (either ampicillin or novobiocin), and whether crude plasmid preparations from the transformants (containing some chromosomal DNA, as well as plasmid DNA) transformed for these markers. However, even in these crude (cleared lysate) plasmid preparations the residual chromosomal DNA transformed with a frequency several orders of magnitude lower than the novobiocin marker from the plasmid (data not shown).

Table 2 shows the results of some of these determinations. Ampicillin resistance was always accompanied by the presence of a plasmid. The same was true of 60 other transformants of other types (data not shown). Thus, the ampicillin marker rarely or never entered the chromosome, or else it was not expressed if it was in the chromosome. The latter hypothesis is improbable, since another ampicillin marker in *H. influenzae* is expressed either on a plasmid or when integrated into the chromosome (1, 2). When the selection was for the chromosomal marker, in no case was there also ampicillin resistance or a plasmid in the transformant. Thus, double transformants from these plasmids were rare. The transformants for the chromosomal marker contained DNA that had become integrated into the chromosome in the usual place, as judged by the normal streptomycin-novobiocin marker linkage shown by lysates of 10 novobiocin transformants of streptomycin-resistant cells (data not shown). Thus, it is improbable that a significant amount of plasmid DNA became integrated along with the chromosomal marker from the plasmid.

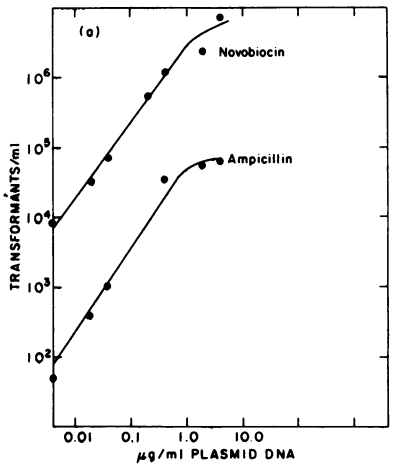

FIG. 3. *Transformation by pNov1 as a function of DNA concentration.*

TABLE 2. *Characteristics of wild-type cells transformed by pNov1*

Selection	No. of transformants studied	Presence of plasmids	Approx size of plasmids	Expression of cloned marker	Resistance to ampicillin
Ampicillin	20	20/20[a]	All like pNov1	5/20[a]	20/20[a]
Novobiocin	17	0/17		17/17	0/17
Ampicillin + novobiocin	13	13/13	All like pNov1	13/13	13/13

[a] Number positive/number tested.

The most remarkable result in Table 2 is that after ampicillin selection in wild-type cells, there were relatively few cases of expression of the cloned marker from the plasmid used for the transformation, although the plasmids in the transformants were the same size as the plasmid used to produce the transformant. Cell-free preparations of all of the transformants were tested for the ability to transform for the unselected chromosomal marker. Such a transformation occurred only in cases where novobiocin resistance was expressed in the transformant (thus, in only 5 of 20 ampicillin transformants from pNov1).

It is clear that even though almost all of the ampicillin transformants lost the chromosomal marker from the plasmid used for the transformation, they still contained chromosomal DNA in their plasmids, as judged by the size of the plasmids inside them and the fact that there was high ampicillin transformation from plasmid preparations of these transformants (data not shown), partly because of specific DNA uptake sites not present in the parent plasmid (9). Thus, the original chromosome material was replaced or altered.

In contrast to the data of Table 2, 10 ampicillin transformants of *rec-1* and 17 ampicillin transformants of *rec-2* were novobiocin resistant.

Nature of the changed chromosomal DNA in pNov1 transformants. The experiments described below were performed to obtain information on the chromosomal insertions in plasmids from wild-type transformants. Plasmids were purified from the following three types of transformants selected for ampicillin resistance: (i) novobiocin-sensitive transformants, (ii) novobiocin-resistant transformants in which novobiocin resistance was not specified by the chromosome of the recipient (as determined by curing the strain of the plasmid), and (iii) novobiocin-resistant transformants in which both the chromosome and the plasmid carried the resistance marker (as determined by curing

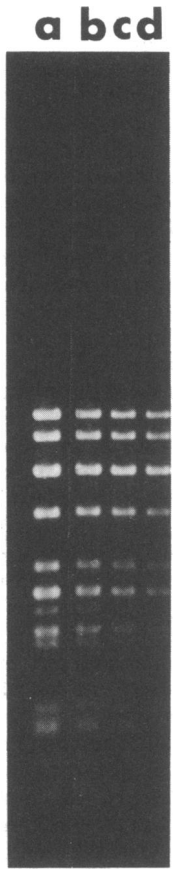

FIG. 4. *Gel electrophoresis of TaqI digests of plasmids from novobiocin-sensitive transformants (lane a), plasmids from novobiocin-resistant transformants of pNov1 (lanes b and c), and pNov1 (lane d). The transformant from which the preparation in lane b was isolated was novobiocin sensitive after curing, and the transformant from which the preparation in lane c was isolated was resistant after curing.*

and high novobiocin transformation by a purified plasmid preparation). As Fig. 4 shows, all of these transformants produced the same *TaqI* restriction digestion pattern, and this pattern was identical to the pattern of the original plasmid, pNov1. This suggested that the chromosomal material in the plasmids of the novobiocin-sensitive transformants could have been replaced by the corresponding part of the DNA of the novobiocin-sensitive recipient. To test this hypothesis, competent wild-type cells which were novobiocin resistant were exposed to purified plasmid DNA from a novobiocin-sensitive transformant; these cells were allowed to grow for a number of generations and then were plated nonselectively. Colonies were picked and transferred with toothpicks to plates with and without novobiocin. Of 151 colonies from cells not exposed to DNA, none was novobiocin sensitive, whereas 218 of the 260 colonies from cells exposed to DNA were novobiocin sensitive. The transformation to novobiocin sensitivity was extraordinarily high, even considering that there were approximately 25 plasmids per cell at the time of exposure. It was clear that the plasmid DNA carried the genetic information for novobiocin sensitivity, which it must have acquired from a recipient novobiocin-sensitive cell.

The results described above implied that if a recipient cell contained the novobiocin resistance marker, an ampicillin transformant transformed by pNov1 should contain only plasmids with the novobiocin resistance marker. A total of 12 ampicillin transformants from a novobiocin-resistant strain that was also resistant to other antibiotics were selected after exposure to pNov1. The plasmids of nine of these transformants were purified and tested for the ability to transform sensitive cells to novobiocin resistance. In all cases, the frequency of transformation to novobiocin resistance was more than three orders of magnitude higher than the frequency of transformation to any other antibiotic resistance conferred by the residual chromosomal DNA of the recipient (data not shown). The DNAs of the other three ampicillin transformants were tested by assaying the DNA of a crude plasmid preparation for transformation to novobiocin resistance before and after the DNA was placed in boiling water for 4 min. This procedure decreased the transformation by chromosomal DNA but increased the transformation by plasmid DNA because of decreased competition (9). We found that the novobiocin resistance marker was present in the plasmids of all three of these transformants (data not shown). The fact that the novobiocin resistance marker was present in the plasmids of 12 of 12 ampicillin transformants from novobiocin-resistant cells, in contrast to the lack of novobiocin resistance in 75% of the pNov1 transformants from novobiocin-sensitive cells (Table 2), is additional evidence that the plasmid could lose a marker by acquiring homologous genetic information from a sensitive recipient cell.

Evidence that the recombination between plasmid and chromosome that results in transfer of a plasmid marker to the chromosome takes place only during the original transformation. It might be imagined that a resident plasmid could recombine with the chromosome at any time during growth of the cells. However, this seems not to be the case. Novobiocin-resistant transformants of strains BC200 and Rd that were exposed to pNov1 and selected for ampicillin resistance were grown from single colonies for about five generations

and then exposed to acridine orange. The survivors of this treatment that were ampicillin sensitive (and thus without plasmids) were tested for sensitivity and resistance to novobiocin. Table 3 shows that in two cases the cells freed of the plasmid had previously acquired the novobiocin resistance marker, but the other 13 isolates were still novobiocin sensitive, as they were before the plasmid transformation. After curing the progeny of each original transformant all were of one type (either novobiocin sensitive or novobiocin resistant); one set of progeny never contained both types. These data suggested that the acquisition of the novobiocin resistance marker by the cell chromosome occurred before the first division after transformation by pNov1 and not at some later time during growth.

A total of 10 novobiocin-resistant *rec-1* and 17 *rec-2* ampicillin transformants were also cured, and we found no evidence of transfer of novobiocin resistance to the cell chromosome, since the cured cells were all novobiocin sensitive.

The state of competence itself is apparently not a sufficient condition for transfer of a marker from plasmid to chromosome, since we obtained evidence that an established pNov1 plasmid did not recombine with the chromosome when the cells were made competent. In this experiment wild-type cells containing pNov1 but lacking the novobiocin marker in the chromosome were grown, made competent, and then regrown in acridine orange for curing. Of 310 cured progeny, none was novobiocin resistant. These data suggested that the established plasmid was sequestered from the chromosome.

Experiments with radioactively labeled plasmids. Plasmid DNA from one of the ampicillin-novobiocin transformants of plasmid pNov1 (Table 2) was isolated from a culture grown with [^{3}H]thymidine, and the profiles of radioactivity and biological activity were determined after sedimentation through sucrose gradients (Fig. 5). It is interesting that transformation for novobiocin and the radioactivity pattern almost coincided, but the ampicillin and ampicillin-novobiocin transformation patterns appeared to be shifted slightly to the faster-sedimenting side of the gradient. This could mean that the open circular form of the plasmid was better for transformation by the inserted chromosomal marker, whereas the closed circular form was better for ampicillin transformation, as shown in the accompanying paper for RSF0885 transformation (9). An alternative hypothesis is that establishment of the plasmid may be favored when there is a rare dimer form.

We can rule out the possibility that chromosomal contamination played a role here, since chromosomal DNA sedimented to the bottom of the tube under the conditions of the experiment. In addition, the amounts of residual chromosomal DNA in cleared lysates were much too low to have any effect on the high frequency of novobiocin transformation (10%).

A peak fraction from a preparation like that

TABLE 3. *Novobiocin resistance and sensitivity in cured progeny of pNov1 transformants of strains Rd and BC200*

Ampicillin transformants[a]		No. of cured progeny tested	Resistance to novobiocin
Strain	Isolate		
Rd	1	208	All −
	2	11	All −
	3	6	All −
	4	51	All −
	5	50	All −
	6	52	All −
	7	52	All −
	8	52	All −
	9	52	All −
	10	50	All +
BC200	1	8	All −
	2	11	All −
	3	3	All +
	4	10	All −
	5	7	All −

[a] Also novobiocin resistant.

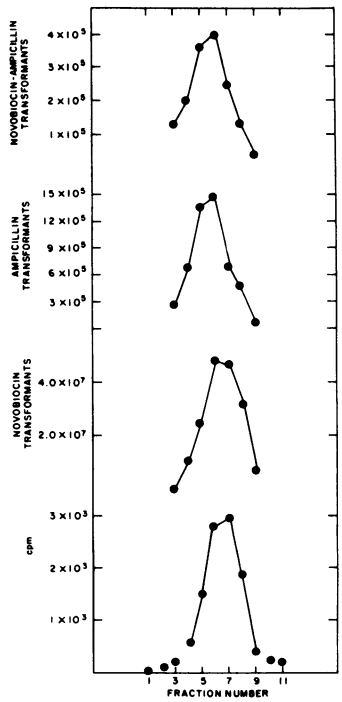

FIG. 5. *Sucrose gradient profiles of radioactively labeled plasmid DNA. Sedimentation was from right to left.*

used for Fig. 5 was used to transform competent cells. To follow the fate of plasmids that entered the cells, samples were withdrawn at different times after DNA uptake was terminated by DNase treatment, and a similar plasmid preparation was made and fractionated on a sucrose gradient (Fig. 6). Again, the peak position of the radioactive counts was close to the peak of novobiocin transformation, whereas the ampicillin and ampicillin-novobiocin transformations formed a peak one fraction nearer the bottom. Also, the radioactivity counts showed that there was a considerable progressive loss of acid-insoluble radioactivity, and there was a small fraction of the material near the bottom of the gradient that exhibited lower biological activity. There was also much loss of biological activity from 0 to 60 min, particularly for ampicillin transformation, but little or no evidence of biologically active DNA near the bottom of the gradient. Nevertheless, at least at early times, much of the input plasmid DNA was recovered in a biologically active form when it was reextracted.

DISCUSSION

RSF0885 contains a defective Tn*A* sequence (4), and thus it is not surprising that the ampicillin marker does not enter the host chromosome by transposition. The fact that RSF0885 transformation to ampicillin resistance is independent of Rec genes (9) is also easy to explain, since it can be assumed that the plasmid enters the cell and can become established without interacting with the recipient chromosome; thus, recombination is not involved. However, the fact that ampicillin transformation from plasmids

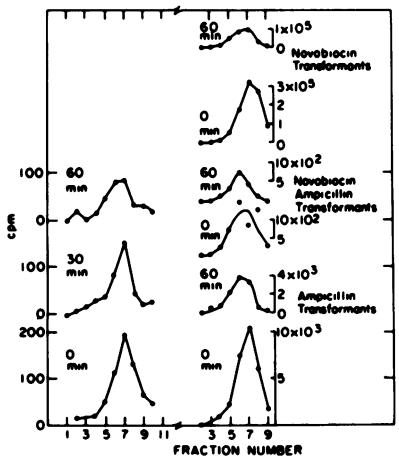

FIG. 6. *Sucrose gradient profiles of radioactively labeled plasmid DNA at different times after entrance into competent cells. Sedimentation was from right to left.*

containing chromosomal insertions is depressed profoundly in Rec⁻ strains (see above) does not have an obvious explanation. One possibility is that the presence of a chromosomal insertion stabilizes the plasmid by pairing at a homologous site on the chromosome. The *rec-2* mutant lacks the ability to permit pairing between transforming DNA and recipient DNA (8), because competent cell DNA in this strain does not form the gaps and tails suitable for pairing (5, 6). The *rec-1* mutant does form such structures, but is prevented from forming recombinants apparently because of local breakdowns at pairing sites (5). Thus, it might be expected that the pNov1 plasmids inside *rec-1* and *rec-2* mutants are more unstable than the pNov1 plasmids in wild-type cells.

We have presented evidence for two types of *rec-1*- and *rec-2*-dependent recombination between a plasmid containing a chromosomal insertion and the chromosome. Both types apparently occur only at a time shortly after a plasmid enters a competent cell. Thus, there is very rare or no recombination between an established plasmid and the chromosome, even when the cells are made competent. In one type of recombination the insertion replaces the homologous region in the chromosome, and the plasmid is usually but not always lost. Our data on the intracellular fate of plasmid radioactivity and markers (Fig. 6) reflect the disintegration of the plasmid and the loss of the ampicillin marker in particular. In the other type of plasmid-chromosome recombination, the plasmid acquires a homologous region of the chromosome in place of part or all of the insertion. We do not believe that these two types usually occur in the same recombination event, since we have not found ampicillin transformants of pNov1 in which the chromosome carries the novobiocin resistance marker and the plasmid carries the corresponding sensitivity marker, as would be expected from reciprocal recombination. Furthermore, the frequency of transformation by the inserted marker of pNov1 is much higher than that of ampicillin (Fig. 3). The ampicillin transformants are more likely to have lost novobiocin resistance altogether than to have this marker in the chromosome of the cell (Tables 2 and 3). When both the plasmid and the cell carry the novobiocin resistance marker, it is likely that one plasmid has recombined with the chromosome and disintegrates after donation of the resistance marker and another plasmid has become established in the cell.

We have considered the possibility that the original transformant of the ligated DNA of pNov1 could contain a mixture of plasmids

carrying novobiocin resistance and sensitivity markers. If this is the case, then the fraction of plasmids carrying sensitivity markers must be relatively low, since no novobiocin-sensitive ampicillin transformants were obtained when recipients already carrying the novobiocin resistance marker were exposed to pNov1 and no novobiocin-sensitive ampicillin transformants of *rec-1* and *rec-2* were found.

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