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August 1982

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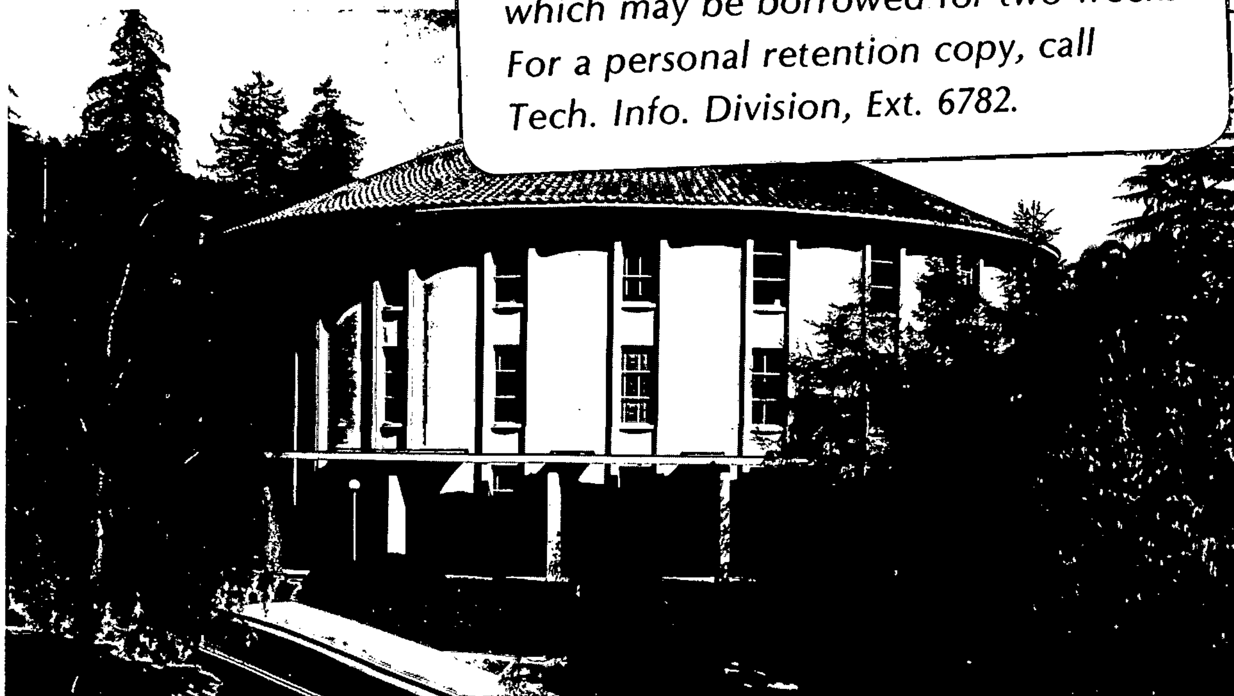

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R-prime Site-Directed Transposon Tn7 Mutagenesis of the Photosynthetic Apparatus in *Rhodopseudomonas capsulata*

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ABSTRACT

Site-directed mutagenesis of the photosynthetic apparatus (PSA) genes in Rhodopseudomonas capsulata is presented utilizing a transposon Tn7 mutagenized R-prime. The R-prime, pRPS404, bears most of the genes necessary for the differentiation of the PSA. Mutagenesis of the R-prime with Tn7 in E. coli, conjugation into R. capsulata, and homologous recombination with the wild-type alleles efficiently generates PSA lesions. Wild-type alleles are lost spontaneously and the Tn7 induced lesions are revealed by subsequent intra-molecular recombination between IS21 insertion elements which bracket the prime sequences in direct repeat. The molecular nature of the intermediates involved in the transposition, recombination, and deletion have been investigated by Southern hybridization analysis. The spontaneous loss of wild-type alleles after homologous recombination with the chromosome may be of general use to other prokaryotic site-directed transposon mutagenesis schemes. The IS21 mediated deletion of the prime DNA is dependent on the Rec A protein in E. coli, generating the parental R-factor bearing one IS21 element.

A genetic-physical map exists for a portion of the prime PSA DNA. When Tn7 is inserted into a bacteriochlorophyll (bch) gene in the R-prime and then crossed into R. capsulata, mutants are produced which accumulate a bch precursor which is in excellent agreement with the existing genetic-physical map. This corroborates our mutagenesis scheme. Mutants arising from Tn7 insertions outside of the genetic map have been isolated. Light harvesting II (LH II) mutations have been isolated; one mutant lacks only the 14 kd polypeptide.

INTRODUCTION

Rhodopseudomonas capsulata is a model organism for the study of anoxygenic photosynthesis because the structure and photochemistry of the PSA have been extensively investigated (for review see Drews, 1978) and because genetic techniques have been developed for this organism (for review see Marrs, 1978; Marrs, 1978b; Marrs et. al, 1980). Bacteriochlorophyll and carotenoid biosynthetic pathways and genes have been characterized (Marrs et. al., 1976; Scolnik et. al., 1980). Reaction center (RC) and light harvesting (LH) polypeptides can be separated and identified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Schumacher & Drews, 1978; Shiozawa et. al., 1980; Youvan et. al., 1982). A gene transfer agent (GTA) similar to a generalized transducing phage has been used for mapping closely linked markers (Solioz et. al., 1975; Yen et. al., 1979) including those encoding bacteriochlorophyll and carotenoid biosynthesis. Large clusters of genes can be mobilized with an R-factor (Marrs, 1981). Using the R-factor mobilization technique, Marrs and coworkers have recently isolated an R-prime, pRPS404, bearing 50 kb of chromosomal DNA that can recombine and correct most lesions in mutants blocked in the differentiation of the PSA. This implies that most or all of the photosynthetic genes are clustered in one 50 kb region of the bacterial chromosome. A genetic map has been determined for approximately twelve genes of the PSA using the gene transfer agent. This map has been recently correlated with a physical restriction map of the R-prime by returning subclones to mutants and checking for recombination (Taylor et. al., 1982; Clark et. al., 1982). Many PSA genes on the R-prime remain to be mapped, presumably including the structural genes for the RC and LH polypeptides. Very recently we have shown (Youvan, 1982) by complementation of enhanced

fluorescence mutants that most or all of the RC and LH genes are carried by the R-prime and that these genes are actively transcribed in *trans* in the homologous organism. Our approach is to use site-directed transposon mutagenesis to determine the coding identity of the DNA outside of the genetically mapped region within the R-prime. The R-prime is mutagenized in *E. coli* with Tn7 and then returned to wild-type *R. capsulata* (Rec^+). Reciprocal homologous recombination trades the mutagenized copy of the PSA genes for the wild-type chromosomal copy. The wild-type alleles, now on the R-prime, delete via intramolecular recombination of insertion elements bracketing the prime DNA in direct repeat. This deletion reveals the mutagenized copy of the PSA genes on the chromosome. The PSA composition of these mutants is screened using a rapid, microprocedure which we have recently described (Youvan et. al, 1982). PSA lesions are correlated with the position of the transposon insertion in the R-prime; a clone of the corresponding mutagenized R-prime has been maintained in a Rec^- *E. coli* library, where it is stable. The target DNA sequence may be subcloned by direct selection for the Tn7 markers (streptomycin, spectinomycin, and trimethoprim). Transpositional properties of Tn7 have been previously characterized using R-factors (Barth et. al, 1976; 1977; 1978), and a restriction map and the terminal sequences of Tn7 have been determined (Lichtenstein and Brenner, 1981; 1982).

Twenty-four different transposon mutagenized R-primes have been conjugated into *R. capsulata*. Four indepently repurified transconjugants were isolated from each cross. Each of these four transconjugants, "cousins", arise from a single clone of donors bearing identical Tn7 mutagenized R-primes. If cousins arising from a single donor show different phenotypes, this is due to the recombinational pathway. The R-prime,

pRPS404, bears a crtD lesion which results in a green colony color that is recessive to the normal red colony color. We find that the cousins from a single donor may differ in the segregation of the green lesion and the transposon lesion, but the PSA lesion, caused by the insertion of the transposon into the chromosome, is identical among cousins which have undergone recombination. For example, when an R-prime bearing a Tn7 insertion in what is known to be the bchC or bchA gene is crossed into *R. capsulata*, mutants accumulate a bacteriochlorophyll precursor characteristic of a bchA lesion. Mutants may be either green or red depending on whether or not the crtD lesion was transfered to the chromosome. Two other transposon mutagenized R-primes cause LH II lesions. One may have an insertion near the 14 kd LH II structural gene, since only this protein is missing from the PSA.

This communication focuses more on the description and characterization of the R-prime site-directed transposon mutagenesis scheme rather than the isolation of new PSA mutants. All steps in the scheme have been investigated by Southern hybridizations using R-factor, R-prime, and Tn7 probes. A mutagenesis model is presented in the Discussion which incorporates both the physical hybridization data and the genetic data.

MATERIAL AND METHODS

(a) Media and Cultivation

E. coli strains were grown in L medium or M9 minimal medium. *R. capsulata* strains were grown chemoheterotrophically on PYE medium or minimal RCV medium at 32 C. Agar plates were sealed with parafilm to maintain humidity and enhance the growth of *R. capsulata* strains. Liquid RCV cultures were grown without shaking and supplemented with 50 mM DMSO,

0.6% dextrose, and 0.5% Na pyruvate (Yen and Marrs, 1977). Photosynthetic growth defective (PSG^-) mutants were screened by making arrayed spots of late-log inoculua on duplicate PYE plates. One plate was incubated aerobically in the dark (permissive for PSG^-) and the duplicate plate was incubated under photosynthetic conditions in an anaerobic jar (BBL GasPak) within a temperature controlled light box (3 mW/cm^2).

(b) Conjugal Matings

R-factors and R-primes were conjugated in spots on well-cured agar plates permissive for the growth of both the donor and the recipient. L agar plates were used for _E. coli_ matings (37 C) and PYE plates were used for matings involving _R. capsulata_ donors or recipients (32 C). Equal volumes of late-log cultures were mixed and 50 μl was spotted and dried. Most matings were incubated four hours, but those involving transposition were incubated longer if the recipient was _R. capsulata_. After mating, bacteria from the spots were resuspended in minimal medium and serially diluted for viable counts and selection of transconjugants. Antibiotics were obtained from Sigma and used at the following levels in micrograms per milliliter: kanamycin 30, streptomycin 15, naladixic acid 50, rifampicin 75, tetracycline 20, and trimethoprim 500.

(c) Chromatophore Analysis

Absorbtion spectroscopy and gel electrophoresis of reaction center and light harvesting polypeptides were performed as recently described (Youvan et. al, 1982). This micromethod utilizes lysozyme and sonication, rather than a French pressure cell, to disrupt the bacteria and release chromatophores. SDS-PAGE minigels can be used to screen large numbers of mutants.

(d) Nucleic Acid Isolation and Analysis

Plasmid DNA was isolated essentially as described in the rapid alkaline-SDS procedure (Birnboim & Doly, 1979) and total cellular DNA was isolated from *R. capsulata* and *E. coli* strains essentially by an existing procedure (Marmur, 1961). DNA samples were digested with restriction enzymes purchased from BRL as specified by the company. Heat-inactivated RNase was added to 1 μg/ml during the digestion. Typically, 0.7% agarose gels were run in a horizontal submarine apparatus (BRL model H4) in E buffer: 40 mM Tris base, 20 mM NaOAc, and 1 mM EDTA (adjusted to pH 7.8 with glacial acetic acid). Analytic gels were 150 ml in volume and Southern gels were 300 ml. Samples were loaded in 1 X E buffer, 0.05% bromophenol blue, and 3% sucrose. Gels were electrophoresed for 16 hours at 2 V/cm.

Nick-translation of DNA probes was performed as previously described (Maniatis et. al, 1975). Bidirectional transfer of DNA to nitrocellulose and hybridization was performed as previously described (Smith & Summers, 1980). Each lane blotted contained approximately 2 μg of DNA, and each filter was probed with approximately 0.5 μg of DNA.

(e) Strain Construction

Antibiotic resistant mutants of the multiple auxotrophic, Rec A^- *E. coli* strain MV12 were selected to serve as recombination free donors and recipients. The rifampicin resistant derivative is denoted NECO100 and the nalidixic acid resistant derivative is denoted NECO125. A strain bearing a chromosomal copy of Tn7, NECO100::Tn7, was prepared by introduction of $RP1_{ts}$::Tn7 into NECO100 from SK1593 via conjugation followed by heat curing of the plasmid in liquid medium at 42 C. Survivors resistant to rifampicin and streptomycin were screened for the loss of R-factor markers (kanamycin and tetracycline) and expression of trimethoprim resistance (a Tn7 marker).

Further confirmation of the loss of the R-factor and transpositon of the Tn7 to the chromosome was indicated by the inability of NEC0100::Tn7 to transfer transposon markers to SB1003 via conjugation in the absence of a conjugative R-factor. Physical evidence that the transposon is present only on the chromosome and that the temperature sensitive R-factor has been cured includes the loss of detectable plasmid bands upon agarose gel electrophoresis of preparations which should yield supercoiled DNA and the hybridization of Tn7 specific probes to two bands indicative of insertion of Tn7 into the chromosomal preferred insertion site described below.

US404 was constructed by conjugal transfer of the R-prime, pRPS404, into NEC0100::Tn7 from HB101. When US404 is crossed with either E. coli or R. capsulata recipients, pRPS404 rescues Tn7 at an efficiency of about 10^{-4} and transfers to recipients with a conjugation efficiency of 10^{-2} per recipient which yields an overall frequency of 10^{-6} per recipient. The NEC0100::Tn7 background is counterselected by auxotrophy and the recipients are selected on plates containing 15 μg/ml streptomycin (supplemented M9 agar for E. coli recipients such as SK2267 or RCV agar for R. capsulata recipients such as SB1003).

T 1

RESULTS

(a) Insertion of Tn7 in the R-prime pRPS404

We have found that the streptomycin resistance marker of transposon Tn7 is expressed in R. capsulata. For the purpose of mutagenizing the conjugative R-prime a $recA^-$ strain of E. coli was constructed (NEC0100::Tn7) bearing a chromosomal Tn7 insertion which is not conjugative. The R prime was conjugated into this strain to make US404 which is NEC0100::Tn7, pRPS404. When this strain is used as a donor and

selection is for streptomycin and kanamycin in the recipient background, then the Tn7 is transferred by insertion into the R-prime. Construction of US404 is described in the Material and Methods.

We have analyzed the sites of Tn7 insertion into the R-prime, pRPS404, by Southern hybridization using Tn7 probes. These experiments require a brief description of the Tn7 restriction map and the subcloned Tn7 probes. Lichtenstein and Brenner (1981; 1982) have recently determined the sequence of the ends of Tn7, mapped restriction sites, and characterized transposition properties. Tn7 has assymetric direct repeats and is 13 kb in length. Eco R1 cleaves the transposon once generating a 8.9 kb left fragment and a 4.3 kb right fragment bearing all of the antibiotic resistance genes. There are two internal Bam H1 sites generating a 0.9 kb internal fragment. In mapping Tn7 insertions with these two enzymes, we expect the loss of the R-prime target fragment which is now part of two new bands. We have obtained Tn7 probes in Col E1 derivatives: pVCT14 bears all of Tn7 and pVCT15 is an Eco R1 deletion derivative containing only the "right" Eco R1 fragment. There is no homology between VCT15 and the left and internal Bam H1 fragments. In Eco R1 digests of Tn7 mutagenized R-primes we expect the VCT14 and VCT15 probes to hybridize to two and one bands, respectively. The same is true for Bam H1 digests with the addition of the small internal fragment self-hybridizing with the VCT14 probe.

In 23 out of 24 Tn7 mutagenized R primes (pRPS404::Tn7) the data are consistent with the insertion of a single transposon in the R-prime and the lack of any detectable rearragements. R-primes bearing multiple insertions have not been observed. With one exception (UAE 91), we observe only one missing R-prime Eco R1 or Bam H1 band in each digest. New bands are generated by the Tn7 and R-prime junctions that hybridize to the VCT14

probe as expected: two fragments hybridize in the Eco R1 digests and two

F 1 fragments plus the internal fragment hybridize in the Bam H1 digests (Fig. 1). In each case only one of the pair of fragments hybridizes with the right-end probe, VCT15. These data may be used to position and orient the Tn7 insertions. Since the restriction fragments involved are large and the maps are at low resolution, positioning is not precise. More accurate data will be available after subclones are made and the R-prime and Tn7 restriction maps are refined. At this time we present the transposon insertion data in tabular form, specifying only the target Bam H1 and Eco

T 2 R1 fragments (Table 2). The insertion in UAE 70 is of particular interest since it is within the genetically mapped region (Taylor et. al., 1982).

(b) Chromosomal Tn7 Insertion Sites

As shown in the previous section, hybridization of a Tn7 probe to blots of mutagenized R-primes reveals two major bands in both Bam H1 and Eco R1 digests since both of the enzymes cleave the transposon into two fragments (neglecting the small internal Bam H1 fragment). When total DNA, rather than episomal DNA, is extracted from strains bearing Tn7 mutagenized R-primes, twice the expected number of hybridizing bands are observed (Fig. 2). Controls indicate that neither Tn7 nor the Col E1 vector has homology with the NEC0125 background bearing the mutagenized R-primes (UAE strains). This phenomenon has been recently explained by Lichtenstein and Brenner. They have observed a chromosomal "hot spot" for Tn7 insertion in the _E. coli_ chromosome at minute 82. Tn7 occupation of this preferred site in a unique orientation was shown to generate two Eco R1 genomic fragments hybridizing with a Tn7 probe. Six out of seven of the genomic Eco R1 digests displayed in Figure 2 possess two bands of approximately 17 kb and 10.5 kb, in close agreement with the previously observed bands in _E. coli_.

When these mutagenized R-primes are crossed into R. capsulata and the genomic blots are repeated, again we observe two extra bands. These bands have a constant size in seven out of seven strains analyzed in figure 2, 10.5 kb and 6.7 kb. Presumably this is an example of a preferred Tn7 integration site in another chromosome. Whether the R. capsulata site has homology with the E. coli preferred site has not been determined. Apart from the insertion of the Tn7 into the preferred chromosomal site, the data in figure 2 are consistent with the interpretation that Tn7 is inserting semirandomly into the R-prime.

F 2

(c) Conjugation of Tn7 mutagenized R-primes into R. capsulata

The next step in the site-directed mutagenesis scheme is to conjugate the Tn7 mutagenized R-primes into wild-type R. capsulata where recombination may introduce the transposon into the PSA genes. We term this process as "clonal" site-directed mutagenesis as opposed to "shotgun" site-directed mutagenesis since the mutagenized R-primes have been purified and stored in E. coli intermediates (UAE library). Shotgun mutagenesis implies a direct cross between US404 and R. capsulata. In the clonal scheme, individual UAE donors are crossed with a strain of R. capsulata bearing a wild-type PSA (SB1003). The resultant R. capsulata transconjugants are designated the UAR strains with a strain number identical to the UAE donor.

Twenty-four UAE strains were mated with SB1003 recipients and selection was made for the transfer of the mutagenized R-prime into the SB1003 background by selection for streptomycin on RCV plates, counterselecting the donors by auxotrophy. Four transconjugants from each cross were repurified several times on RCV medium containing streptomycin. These independently repurified transconjugants arising from a mating with a

clonal donor are termed "cousins". Different cousins are designated by a lower case letter following the strain number. Matings are sufficiently efficient (10^{-2} per recipient) that the probability that any two cousins are siblings is far less than 10%. This estimate is made based on the worst case where all transconjugants were generated at the begining of the mating and multiplied exponentialy until the mating was disrupted. Cousins may or may not have identical phenotypes. This depends on the particular recombination events which occur in the Rec^+ merozygote.

We have investigated the fate of the Tn7 mutagenized DNA in R. capsulata by comparing genomic blots of the UAE strains with the corresponding UAR strains probed with Tn7. Figure 3 clearly reveals that the position of the Tn7 relative to the adjacent prime restriction sites is unchanged. The integrity of the Tn7 mutagenesis site is maintained in the Rec^+ background. Recombinational events which may have incorporated the mutagenized prime into the chromosome have preserved the adjacent prime restriction sites. We can estimate the plasmid copy number by comparing the intensities of the preferred insertion bands in the chromosome with the bands corresponding to insertion in the PSA DNA. In most cases it appears that the PSA insertion signal is two to four times greater than the chromosomal preferred site signal, hence we must be observing one to three copies of the mutagenized R-prime sequences in addition to the copy which has recombined into the chromosome. That this is indeed the case will be shown in later sections by genomic blots using the R-prime as a probe.

(d) Phenotypes of the UAR strains

Cousins, independently repurified transconjugants arising from clonal donors, have either identical phenotypes or no detectable change in the PSA phenotype. No selection was made in the picking and repurification

of mutants, ie. typical colonies were picked. Identical phenotypes among cousins is very strong evidence that our mutagenesis scheme is indeed site-directed and that the mutant phenotype is the result of the Tn7 insertion. Mutants may be either green or red depending on whether or not the R-prime crtD lesion was successfully recombined into the chromosome. An exception to this rule occurs when the Tn7 lesion reduces the overall expression of carotenoids. Of the twenty-four different mutagenized R-primes crossed into SB1003, we have found PSG$^-$ cousins in six of the UAR series. These mutations and the positions of the transposons relative to the R-prime Eco R1 and Bam H1 bands are listed in table 3.

T 3

Four out of four of the independently repurified transconjugants from the UAE70 X SB1003 cross have identical PSA mutations. All of these cousins lack the RC and LH polypeptides and accumulate a bacteriochlorophyll (bch) precursor which absorbs at 665 nm, characteristic of a lesion in the bchA gene. Results from absorption spectroscopy and SDS-PAGE of chromatophores from the UAR strains are summarized in Table 3. The UAE70 Tn7 insertion maps to the Bam C - Eco H fragment of pRPS404. In the physical-genetic map recently determined (Taylor et. al., 1982), this lesion maps in the bchC gene which is immediately adjacent to the bchA gene. Marrs (personal communication) has previously observed lesions which map via GTA to the bchC gene and cause a bchA phenotype. Presumably, in both cases, the lesion in the bchC gene has a polar effect on the expression of the bchA gene. In the case of the transposon mutant UAR70 this is expected if the insertion is between the promoter and the bchA gene.

Four out of four of the cousins arising from the UAE115 donor have identical lesions in the LH II antenna. Three are green and one is red.

These mutants have abnormally low absorption at 800 nm and higher absorption at 880 nm. The visible spectra are virtually superimposible with the exception of the difference in the carotenoids associated with the crtD lesion. All of these cousins lack the 14 kd LH II polypeptide. The 14 kd polypeptide may be involved in the assembly of the LH II antenna by facilitating the binding of bacteriochlorophyll to the 8 and 10 kd polypeptides which generates the 800 nm absorption band. The UAE 115 Tn7 insertion maps to the Bam E - Eco E fragment which is outside of the known genetic map. One of four of the UAR18 cousins, UAR18f, is PSG$^-$ and has an insertion in the Bam E - Eco E fragment. This insertion maps approximately 2 kb away from the site in the UAR115 mutants. UAR18f has almost no absorption at 800 nm and lacks only the 14 kd LH II polypeptide. It has wild-type carotenoid coloration and no other detectable PSA lesions. These data suggest tha the Bam E - Eco E fragment contains genes affecting the LH II antenna. We believe that the UAE18 insertion may be near the structural gene for the 14 kd polypeptide.

Five other mutants in table 3 lack bacteriochlorophyll and all PSA polypeptides. These Tn7 lesions map outside of the genetic map. Some may be in other bacteriochlorophyll biosynthetic genes, the inactivation of which turns off the expression of the PSA. Testing four cousins from each cross and finding no altered PSA phenotypes does not rule out the possibility that the position of the transposon in the DNA is mutagenic. As will be shown later, prime DNA bearing the Tn7 insertion may be deleted before recombination with the chromosome. We have found that infrared photography of colonies can be used to screen for mutants lacking reaction centers. These mutants are highly fluorescent in the near infrared (Youvan et. al., 1982). This technique will facilitate the screening of large numbers of

cousins so that less frequent recombination events leading to the loss of reaction centers can be observed.

(e) Rec A^+ Dependent Deletion of Prime DNA mediated by IS21 Elements

In the previous section we have demonstrated that both the transposon lesion and the _crtD_ lesion may be expressed in _R. capsulata_ transconjugants. Apparently, the wild-type alleles are not complementing these lesions in _trans_ as expected from a reciprocal homologous recombination. One explanation for the loss of the wild-type phenotype after recombination with the mutagenized R-prime is that the chromosomal wild-type alleles, transiently on the R-prime, have been deleted. This is not due to the loss of the entire R-prime since the kanamycin marker is stable for at least two hundred generations without selection. Mobilization of DNA using the R-factor pBLM2 results in direct repeats of IS21 flanking the prime DNA (Clark et. al., 1982). This suggests a mechanism for the deletion of prime DNA and loss of wild-type alleles. Intramolecular recombination between the IS21 elements should regenerate the parent R-factor pLM2 bearing one IS21 element and a circular piece of DNA bearing the prime DNA and the other IS21 element. The circular prime DNA is subsequently lost since it is not a replicon. The structure of pLM2 and pBLM2 regarding the tandem duplication of IS21 elements is analogous to the structural changes that have been well-characterized for R68 and R68.45 which also result in enhanced chromosomal mobilizing ability (Rieb et. al., 1980).

To test for prime deletion we conjugated pRPS404 from a Rec A^- background (HB101) into two isogenic strains: SK2267 (_recA_$^-$) and SK1593 (_recA_$^+$). Transconjugants were selected by kanamycin and the donor was conterselected by auxotrophy on M9 medium. These strains were repurified

two times on M9 medium containing kanamycin. In the E. coli background, intermolecular recombination between the R-prime and the chromosome is eliminated since there is no DNA homology as (assayed by Southern hybridizations). Plasmids were prepared from these strains by the alkaline-SDS procedure described in the Methods section. Plasmids were subjected to restriction digestion and agarose gel electrophoresis. 18 independently repurified strains of SK2267 (pRRS404) are stable as assayed by Eco R1 restriction (data not shown). 15 out of 15 strains of SK1593 (pRPS404) are in various stages of prime deletion (Fig. 4). This DNA has been replicated approximately 50 generations between conjugation and plasmid extraction. We interpret the non-stoichiometric ratios among bands to represent mixed populations of the deleted R-factor and undeleted R-prime. The most intense bands map to the R-factor (Eco C, G, and K). The ratio of the intensities of the Eco G band to the Eco F or Eco H bands is a measure of the ratio of total plasmids to undeleted plasmids. The same DNA samples from SK1593 (pRPS404) were subjected to a Hind III and Sal I double digest. Analogous to R68 and R68.45, a 4640 bp fragment is characteristic of one IS21 element (pLM2) and a 6760 bp fragment is characteristic of two IS21 elements (pBLM2). All plasmids analyzed were deleting to pLM2 as indicated a 4640 bp Hind III - Sal I fragment (Fig. 5).

F 4

F 5

Deletion of pRPS404 to parental R-factors has also been observed in the homologous organism. Similar to the E. coli deletion experiments described above, pRPS404 was conjugated from HB101 into SB1003. R. capsulata transconjugants were selected on RCV medium containing kanamycin and the E. coli donor was counterselected by auxotrophy. After two stages of repurification, DNA was extracted by the modified alkaline-SDS procedure and only R-factor bands were observed with Eco R1 digestion (data not

shown). These digests are obscured by endogenous plasmid bands. However the fusion of the R-factor Eco A and Eco B bands into a higher molecular weight band characteristic of pLM2 was observed. The following experiment was designed to avoid the problems generated by the endogenous plasmid bands. Kanamycin resistant plasmids were rescued from the UAR strains by conjugation into a Rec A$^-$ background, NECO125. NECO125 strains bearing plasmids were selected by naladixic acid and kanamycin. It is critical to note that up to this point the UAR strains have been maintained under constant streptomycin selection. For matings, the UAR strains were subcultured without antibiotic selection. We have observed that kanamycin resistance is stable in the UAR strains for at least 200 generations without selection but that the level of resistance to streptomycin is significantly reduced after subculturing without selection for streptomycin. Strains originally resistant to 15 μg/ml of streptomycin on plates form only pin-point colonies on these plates after the colonies have been subcultured in the absence of streptomycin. This is genetic evidence

F 6

for the deletion of the multi-copy mutagenized R-prime Tn7 marker. Figure 6 demonstrates that this is due to IS21 mediated deletion of the prime DNA Of the 12 plasmids independently isolated from PSG$^-$ UAR strains, the characteristic Hind III - Sal I fragment indicates that 7 plasmids have deleted to pLM2 and 5 plasmids have deleted to pBLM2. Two lanes display plasmids that upon further analysis were found to have some undeleted prime sequences. An alternative explanation is that 12 plasmids deleted to pLM2 and 5 of them underwent a second transposition event generating another copy of IS21. In a later section we will show that deletion of the chromosomal copy of the PSA genes occurs immediately after recombination but that other copies of the mutagenized R-prime do not delete until the

streptomycin selection is discontinued. This is due to a gene-dosage effect giving a selective advantage to bacteria containing undeleted R-primes and multiple copies of Tn7.

(f) Analysis of Tn7 Insertions into the R. capsulata Chromosome

Southern hybridizations to total DNA from the UAR strains blotted bidirectionally and probed with the pRPS404 and Tn7 are shown in the left

F 7 and right panels of figure 7. With the R-prime probe, we expect that the target PSA restriction fragment will be missing in each lane due to the insertion of the transposon which has internal Bam H1 and Eco R1 sites. This is indeed the case and is evidence for recombination, incorporation of the Tn7 lesion into the chromosomal PSA genes and loss of the wild type alleles. This figure can be compared to the insertions listed in Table 2 to verify that the target fragment is missing from the blot probed with the R-prime. Of particular interest are strains UAR18f, UAR 70e, and UAR 115h, all of which possess mutant PSA phenotypes. Both UAR18f and the entire UAR115 series have LH II lesions with reduced 800 nm absorption and loss of the 14 kd polypeptide. Tn7 was mapped to the Bam E - Eco E fragment in the corresponding donors: UAE18 and UAE115, and these fragments are missing in the genomic blot (lanes c and i, respectively). UAR70e accummulates a bch precursor characteristic of a bchA lesion. This is consistent with the insertion of Tn7 into the Bam C - Eco H fragment in the genetically mapped region. The R-prime probed blot (lane g) reveals that both of these fragments are missing. Eco A, Eco B, Bam A, and Bam B fragments characteristic of the undeleted R-prime are present in all of the UAR strains in figure 7 except UAR19e (lane d). All of the stains have been under continuous streptomycin selection which selects against deletion of prime sequences bearing Tn7 insertions. The Tn7 insertion in UAR19e is in

the Bam A - Eco G fragment which is part of the R-factor, hence deletion of the prime sequence does not affect the level of streptomycin resistance. Most of the signal in this blot is due to undeleted R-primes, but we know that there is sufficient sensitivity to observe any chromosomal copies of the fragments since the prime-chromosome junction fragments can be observed in the genomic digest of SB1003 cut with Eco R1 and probed with pRPS404. In the right half of figure 7 the same samples have been probed with Tn7 (VCT14). Eco R1 and Bam H1 digests of US404 (sample a) reveal hybridizing bands characteristic in size of the Tn7 insertion into the E. coli preferred site at minute 82. Sample b is total DNA from SB1003 which shows no cross-hybridization with the Tn7 probe or Col E1 vector. All of the UAR strains have three common Bam H1 bands: the upper two common bands are characteristic of the chromosomal preferred insertion site and the lower common band is the internal Bam H1 fragment from Tn7. The two common bands in the Eco R1 digests are also due to the chromosomal preferred insertion site. Other bands in each digest are characteristic of the junction fragments between the ends of Tn7 and the target fragment.

(g) Deletion of Prime Sequences in the Absence of Streptomycin Selection

Assuming that deleted and undeleted plasmids transfer with equal frequencies, the analysis of deleted plasmids in the UAR strains by conjugal transfer into a Rec A$^-$ E. coli background suggests that approximately 80% of the mutagenized R-primes have deleted. However, the experiments described in the preceding section utilizing R-prime probed total UAR DNA suggests that a much smaller proportion of the R-primes have been deleted. This apparent inconsistentcy is resolved by comparing blots of UAR strains which have been under continuous streptomycin selection to

F 8 strains subcultured without streptomycin selection. Figure 8 reveals that

the strains subcultured without streptomycin delete to the parental R-factor as indicated by the loss of the Eco A and Eco B bands and appearance of a higher molecular weight band predicted from prime deletion and fusion of the Eco A and Eco B arms. Of the three strains tested in this experiment, two bear only deleted plasmids (UAR18f and UAR115h) and one strain contains a mixture of deleted and undeleted plasmids (UAR70g). Presumably, R-primes in UAR70g are still in the process of deleting. IS21 mediated deletion of the unselected prime sequence explains the deleted plasmids observed in the conjugal rescue experiment. In order to mate with a streptomycin sensitive recipient, the UAR donors were subcultured without streptomycin and apparently deleted at that time.

DISCUSSION

Our scheme for site-directed mutagenesis in R. capsulata using an R-prime bearing the genes for anoxygenic photosynthesis is shown in figures

F 9

9 and 10. The genetic data and physical Southern hybridization data supporting this model have been presented above. Transposon Tn7 is inserted into pRPS404 in a Rec A$^-$ E. coli background and then the R-prime::Tn7 is conjugated into the homologous organism where recombination may transfer the mutagenized DNA into the chromosome. IS21 mediated intramolecular recombination on the recombinant R-prime subsequently deletes the wild type alleles.

The following is a brief summary of the clonal site-directed transposon mutagenesis scheme. US404 contains a chromosomal copy of Tn7 in the preferred insertion site at minute 82 and the R-prime, pRPS404, bearing the PSA genes. All of the E. coli strains used in this scheme are Rec A$^-$. The prime sequence of pRPS404 specifies most of the genes necessary for the

differentiation of the PSA and is marked by a crtD lesion which results in the accumulation of a green colored carotenoid. Tn7 is semirandomly inserted into pRPS404 and conjugated into a recipient, SK2267, by selection for streptomycin, and the donor is counterselected. The overall efficiency is 10^{-6} per SK2267 recipient. Twenty-four mutagenized plasmids in the UA library have mapped Tn7 insertions. Insertions are semirandom but there is some preference for the Bam E fragment. SK2267 was selected because it is a highly efficient recipient, but it cannot be counterselected in crosses with SB1003. The UA library was transferred into a more auxotrophic strain, NECO125, by selection for streptomycin and naladixic acid. Next, individual UAE strains (NECO125, pRPS404::Tn7) are crossed with wild-type R. capsulata strain SB1003 and merozygotes are selected by streptomycin. These merozygotic colonies develop green sectors as the crtD lesion is recombined into the chromosome and the wild-type allele is deleted. The frequencies of these events are sufficiently high that the perimeter of the colonies becomes entirely green. Upon repurification, stable red and green colonies result. Independently repurified transconjugants stemming from one donor are termed cousins. Since an E. coli library was used in this scheme the mutagenesis is termed "clonal". Cousins bear mutagenized R-primes with identical Tn7 insertions but their phenotypes may differ depending on whether or not the crtD lesion and the Tn7 lesion are integrated into the chromosome. Four cousins arising from a single cross are shown in figure 9. Let us assume that the Tn7 insertion is in a gene essential for photosynthetic growth. Different lower case letters designate cousins from this cross: a) PSG^{-}, green; b) PSG^{+}, red; c) PSG^{-}, red; d) PSG^{+}, green. The Tn7 preferred insertion site is occupied in all of the strains in figure 9 because transposition to this site occurs very soon after any Tn7 bearing

plasmid is introduced into the background. There is no detectable phenotypic effect due to this insertion in either *E. coli* or *R. capsulata*. Figure 9 is an oversimplification of the recombination and deletion processes in that only one copy of the mutagenized R-prime is shown; only the R-prime which will recombine is depicted. After recombination, the wild-type alleles are deleted from this particular recombinant R-prime and the *crtD* and Tn7 mutations in the chromosome are phenotypically expressed. Other copies of mutagenized R-primes do not delete until the streptomycin selection is terminated and the bacteria are subcultured (Fig. 10). While under selection, multiple copies of Tn7 yield higher levels of antibiotic resistance, hence undeleted plasmids are selected.

F 10

Ruvkun and Ausubel (1981) proposed a general scheme for site-directed mutagenesis using homologous recombination with transposon mutagenized plasmids. Recombined plasmids bearing wild-type alleles must be excluded to reveal the mutagenized chromosomal gene. This requires a second cross in which a second, incompatible plasmid is selected. This was our original plan until we observed the spontaneous deletion of wild-type alleles. We have shown that the deletion is due to intramolecular homologous recombination between IS21 elements flanking the prime sequence. This process is dependent on the Rec A protein in *E. coli* and, presumably, a similar protein in *R. capsulata*. Selection for streptomycin selects against deleted mutagenized R-primes because of a gene-dosage effect increasing the level of resistance with increasing numbers of transposons. This enhances recombination with the chromosome since multiple copies of the mutagenized alleles are maintained. After recombination with the chromosome, the wild-type alleles, transiently carried by an R-prime, are deleted via intramolecular recombination between the flanking IS21

elements. This deletion is immediate since the prime bears no resistance marker. An alternative explanation is that this plasmid is lost by competition with the other R-primes which contribute to the antibiotic resistance. Remaining prime sequences delete after the antibiotic is removed and the mutants are subcultured. Under kanamycin selection alone, merozygotic colonies of SB1003 (pRPS404) develop only infrequent and narrow green sectors since there is no selection against strains bearing deleted R-primes.

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REFERENCES

Barth, P. T., Dalta, N., Hedges, R. W., and Grinter, N. J. (1976) J. Bact. 125, 800-810.

Barth, P.T. and Grinter, N.J. (1977) DNA Insertion Elements, Plasmids, and Episomes, p. 675-677. Bukhari, A. I., Shapiro, J. A., Adhya, S. L., eds. Cold Spring Harbor Laboratory.

Barth, P. T., Grinter, N. J., and Bradley, D. E. (1978) J. Bact. 133, 43-52.

Birnboim, H. C. and Doly, J. (1979) Nucl. Ac. Res. 7, 1513-1523.

Clark, W. G., Schivily, J. M., Taylor, D. P., Cohen, S. N. and Marrs, B. L. (1982) J. Bact., submitted.

Drews, G., Dierstein, R. and Schumacher, A. (1976) FEBS Lett. 68, 132-136.

Drews, G. (1978) Current Topics in Bioenergetics 8, 161-207.

Lichtenstein, C. and Brenner, S. (1981) Molec. Gen. Genet., 183, 380-387.

Lichtenstein, C. and Brenner, S. (1982) Nature, 297, 601-603.

Maniatis, T., Jeffrey, A. and Kleid, D. G. (1975) Proc. Nat. Acad. Sci. USA 72, 1184-1188.

Marmur, J. (1961) J. Mol. Biol. 3, 208-218.

Marrs, B. L.and Yen, H.C. (1976) J. Bact. 126, 619-629.

Marrs, B. L. (1978) Current Topics in Bioenergetics 8, 261-294.

Marrs, B. L. (1978) The Photosynthetic Bacteria, P. 873-883.

Marrs, B. L., Kaplan, S., and Shepard, W. (1980) Meth. Enzym. 69, 29-37.

Marrs, B. L. (1981) J. Bact., 146, 1003-1012.

Mindch, L., Cohen, J. and Weisburd, M. (1976) J. Bact. 126, 177-182.

Rieh, G., Holloway, B. W. and Puhler, A. (1980) Genet. Res. 36, 99-109.

Ruvkun, G.B. and Ausubel, F. M. (1981) Nature (London) 289, 85-88.

Sakanyan, V. A., Yakubov, L. Z., Alikhanian, S. I., and Stepanov, A. I. (1978) Molec. Gen. Genet. 165, 331-341.

Sato, M., Staskawicz, B. J., Panopoulos, N. J., Peters, S., and Honma, M. (1981) Plasmid, 6, 325-331.

Schumacher, A. and Drews, G. (1978) Biochem. Biophy. Acta 501, 183-194.

Schumacher, A. and Drews, G. (1979) Biochem. 547, 417-428.

Scolnik, P. A., Walker, M. A., Marrs, B. L. (1980) J. Biol. Chem. 255, 2427-2482.

Scolnik, P.A., Zannoni, D., and Marrs, B. L. (1980) Biochim. Biophys. Acta 593 230-240.

Shiozawa, J. A., Cuendet, P. A., Drews, G., and Zuber, H. (1980) Eur. J. Biochem. 111, 455-460.

Smith, S. E. and Summers, M. D. (1980) Analy. Biochem. 109, 123-129.

Solioz, M., Yen, H.C., and Marrs, B. L. (1975) J. Bact. 123, 651-657.

Taylor, D. P., Cohen, S. N., Clark, W. G., Marrs, B. L. (1982) J. Bact., submitted.

Yen, H.-C. and Marrs, B. (1977) Biochem. Biophys. 181, 411-418.

Yen, H.-C., Hu, N.T., and Marrs, B. L. (1979) J. Mol. Biol. 131, 157-168.

Youvan, D. C., Zsebo, K. and Hearst, J. E. (1982) J. Bact., submitted.

TABLE LEGENDS

Table 1. Bacterial Strains and Plasmids. * Note: UAR#l strains are the repurified transconjugants from the corresponding UAR# merozygotes resulting from crosses between a particular UAE# donor and SB1003 recipients. The lower case letter (l) after the strain number (#) designates a particular, independently repurified transconjugant. A set of independently repurified transconjugants arising from the same donor and bearing the same strain number are termed "cousins". Abbreviations: ampicillin (Am), kanamycin (Km), naladixic acid (Nl), rifampicin (Rf), spectinomycin (Sp), streptomycin (Sm), trimethoprim (Tm), chromosomal mobilizing ability (cma), and photosynthetic apparatus (PSA).

Table 2. Strains Bearing Mutagenized R-Primes with Mapped Tn7 Insertions. The UAE strains are NEC0125 backgrounds containing Tn7 mutagenized pRPS404 R-primes. The position of the Tn7 insert is designated by the Bam H1 and Eco R1 fragments of the R-prime which bear the insert. The R-prime restriction fragments are designated by alphabetic upper case letters starting with the largest fragment which is designated "A". Key: ND, no detectable pertubation of the pRPS404 restriction pattern (due to lack of resolution in the screening gel for large and small fragments or commigration of junction fragments and an R-prime fragment); NT, no test.

Table 3. Phenotypes of R. capsulata UAR Strains Defective for Photosynthetic Growth. Twenty-four UAE strains with mapped Tn7 insertions were crossed into SB1003 and four cousins were selected from each cross. Of the 96 cousins assayed, 13 were PSG$^-$ and further analyzed by absorption spectroscopy and SDS-PAGE. The PSA lesions are listed in terms of missing

polypeptides and altered infrared absorption. The UAR70 series is of particular interest since the Tn7 insertion in the Bam C - Eco H fragment maps near or within the bchA and bchC genes, and the mutant phenotype (a bacterichlorophyll precursor absorbing at 665 nm) is characteristic of a bchA lesion. UAR18f is lacking only the 14 kd LH II polypeptide suggesting that this LH II gene may be in the Bam E - Eco E fragment.

FIGURE LEGENDS

Figure 1. Southern Hybridization of Tn7 Probes to Mutagenized R-Primes. Transposon Tn7 mutagenized plasmids were isolated from the UAR strains, digested with either Eco R1 or Bam H1 restriction endonucleases, and subjected to electrophoresis on a 0.7% agarose gel. The gel was blotted by the bidirectional procedure and probed with the entire transposon (pVCT14 probe) or the antibiotic resistant Eco R1 fragment from Tn7 (pVCT15 probe). These hybridization patterns are consistent with single insertions of Tn7, see text. Samples: M, Phage lambda DNA Hind III markers (molecular weight in kilo basepairs is designated to the left); R, R-prime pRPS404; a, UAE70; b, UAE78; c, UAE81; d, UAE91; e, UAE131.

Figure 2. Insertion of Tn7 Into a Preferred Site in the Chromosomes of _E. coli_ and _R. capsulata_. Total DNA was extracted from nine strains of _E. coli_ and _R. capsulata_ (UAE and UAR strains, respectively) bearing Tn7 mutagenized pRPS404. DNA was digested with Eco R1, electrophoresed on a 0.7% agarose gel, transferred bidirectionally to nitrocellulose, and hybridized with nick-translated Tn7-specific probes. Left panel: VCT14 probe bearing the intact transposon; right panel: VCT15 probe bearing the antibiotic marked end of Tn7 borne on the terminal Eco R1 fragment. Lane M, Hind III lambda DNA markers: 24, 9.8, 6.9, 2.3, 2.0 kb. Lanes a-i, total DNA from _E. coli_ strains digested with Eco R1: lane a, NECO100::Tn7; b, UAE18; c, UAE19; d, UAE22; e, UAE39; f, UAE67; g, UAE70; h, UAE78; i, UAE115. Lanes j-s, total DNA from _R. capsulata_ strains digested with Eco R1: lane j, SB1003; k, UAR18f; l, UAR19e; m, UAR22h; n, UAR39f; o, UAR67h; p, UAR70e; q, UAR78e; r, UAR115h; s, U23. E, fragments generated by preferential Tn7 insertion at minute 82 in the _E. coli_

chromosome (17 and 10.5 kb). R, fragments generated by preferential Tn7 insertion at a unique site in the R. capsulata chromosome (10.5 and 6.7 kb).

Figure 3. Comparison of Tn7 Insertions in the UAE and UAR Strains. Total DNA was extracted from five E. coli strains (UAE) bearing Tn7 mutagenized R-primes and corresponding R. capsulata strains (UAR). DNA was digested with Eco R1, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with the nick-translated VCT14 probe bearing the entire Tn7 sequence. Lanes are arranged so as to demonstrate the stability of the transposon insertion in the prime DNA. Lane o was mistakenly digested with Bam H1. Lane M, Hind III lambda DNA markers: 24, 9.8, 6.9, 2.3, 2.0 kb. Lane a, UAE18; b, UAR18f; c, U23; d, UAE22; e, UAR22h; f, UAE39; g, UAR39f; h, UAR39h; i, UAE70; j, UAR70e; k, UAR70f; l, UAR70g; m, UAR70h; n, UAE115; o, UAR115e; p, UAR115f; q, UAR115g; r, UAR115h. Bands marked E and R refer to the fragments generated as a result of Tn7 insertion into the preferred chromosomal sites in E. coli and R. capsulata, respectively.

Figure 4. Deletion of the Prime Sequence from pRPS404 in Rec A$^+$ E. coli. The R-prime pRPS404 was conjugated from HB101 (recA$^-$) into SK1593 (recA$^+$) and SK1593 (pRPS404) transconjugants were repurified by streaking for isolated colonies two times. Plasmids were extracted from fifteen of these strains and analyzed by Eco R1 digestion and electrophoresis on a 0.7% agarose gel (stained with ethidium bromide). Higher than stoichiometric amounts of the R-factor specific bands are seen in all of the samples. Lanes a, b, c, d, g, i, l and m demonstrate that some of these

strains are deleted almost entirely to the parental R-factors. Other lanes: e, f, h, j, k, m and o reveal various mixtures of R-factor and R-primes. Lane M, Hind III lambda markers: 24, 9.8, 6.9, 2.3 and 2.0 kb. Lane R, R-prime pRPS404 with bands labeled to the left (A-L, with R-factor specific bands underlined). Δ refers to the deleted product of the Eco A and Eco B bands.

Figure 5. Deletion of the R-prime pRPS404 to the Parental R-factor pLM2. These plasmid samples are identical to those in figure 4, except in this case the DNA has been digested with Hind III and Sal I. Digests of the parental R-factors pBLM2 (two IS21 elements) and pLM2 (one IS21 element) are shown in lanes B and L . Bands labeled L and B refer to the Hind III - Sal I fragment (4640 kb) spanning the IS21 element in pLM2 and the Hind III - Sal I fragment (6760 kb) spanning the tandem IS21 elements in pBLM2. Lanes a to o reveal that plasmids from all of the SK1593 (pRPS404) strains are deleting to pLM2. Lane M, Hind III lambda markers.

Figure 6. Deleted R-primes Rescued from the UAR Strains. Thirteen PSG$^-$ UAR strains were conjugated with NECO125 and kanamycin resistant transconjugants were selected. Plasmids were extracted from one transconjugant in each of the thirteen crosses. Each DNA sample was digested with Eco R1 and electrophoresed on a 0.7% agarose gel (ethidium bromide stain). Both pLM2 and pBLM2 deletion products were observed. Lanes B and L and bands marked B and L are identical to those described in the legend to figure 5. Lanes a and b contain samples identical to those displayed in figure 5. Lanes c - o contain digests of plasmids rescued from the UAR strains: c, UAR18f; d, UAR22h; e, UAR39f; f, UAR39h; g, UAR70e; h,

UAR70f; i, UAR70g; j, UAR70h; k, UAR115e; l, UAR115f; m, UAR115g; n, UAR115h; o, UAR219e. In two cases, plasmids bear prime sequences: UAR22h and UAR70h.

Figure 7. Analysis of Tn7 Insertions in the PSA Genes of R. capsulata. Total DNA prepared from R. capsulata strains which received different Tn7 mutagenized R-primes by conjugation (UAR strains) were subjected to digestion with Eco R1 or Bam H1 restriction endonucleses, electrophoresed on a 0.7% agarose gel, and blotted bidirectionally to nitrocellulose. One filter was hybridized with an R-prime specific probe prepared by nick-translation of pRPS404 DNA; the other with Tn7 specific probe similarly prepared from pVCT14 DNA. Left panel: R-prime specific probe. Letters A-K to the left of the blot and letters A-M on the right refer to the Bam H1 and Eco R1 restriction fragments from pRPS404. Lanes marked M refer to Hind III lambda markers with the fragment sizes in kb listed on the left side of the right panel. As each UAR strain is listed, the strain number will be followed by two upper case letters which designate the R-prime Bam H1 and Eco R1 restriction fragment, respectively, into which the transposon has inserted (also, see Table 2). Strain UAR87e has no detectable PSA lesion and the corresponding chromosomal fragment is intact. Sample a, US404; b, SB1003; c, UAR18f (E,E); d, UAR19e (A,G); e, UAR39f (D,F); f, UAR67h (F,F); g, UAR70e (C,H); h, UAR87e (F,F); i, UAR115h (E,E).

Figure 8. Deletion of Prime Sequences in UAR Strains Subcultured Without Streptomycin Selection. Total DNA from E. coli donors (UAE strains) and the corresponding R. capsulata mutants (UAR strains) was digested with

Eco R1 or Bam H1, electrophoresed on a 0.7% agarose gel, blotted bidirectionally to nitrocellulose and probed. The left panel was probed with the R-prime pRPS404 and the right panel was probed with the R-factor pBLM2. Samples alternate in the order: UAE donor, UAR mutant (cultured continuously under streptomycin selection), and UAR mutant (further subcultured without streptomycin selection). Deletion of the prime sequence via IS21 mediated homologous recombination is indicated by the loss of the two largest Eco R1 and Bam H1 bands (A and B, in both digests) and the generation of a larger band (designated Δ). This is due to the loss of Eco R1 restriction sites in the prime DNA. Samples on the left side of each panel were digested with Eco R1 and the samples on the right side of each panel were digested with Bam H1. Symbols following the UAR strain number designate whether streptomycin was present in the subculture from which the total DNA was extracted: a, UAE18; b, UAR18f (+); c, UAR18f (-); d, UAE115; e, UAR115h (+); f, UAR115h(-); g, UAE70; h, UAR70e (+); i, UAR70g (-); j, US404; k, SB1003.

Figure 9. A Model for the Mechanism of Clonal Site Directed Transposon Mutagenesis in Rhodopseudomonas capsulata. The R-prime pRPS404 is mutagenized in a Rec A^- background of E. coli and then transferred to wild-type R. capsulata by conjugation where homologous recombination may exchange the mutagenized PSA sequences for the chromosomal copy. IS21 mediated intramolecular homologous recombination deletes the wild type alleles from the R-prime. Four possible outcomes are shown for independently repurified transconjugants arising from a single donor. These "cousins" possess identical Tn7 induced lesions in the PSA if the transposon has recombined into the chromosome. The independently

segregating crtD lesion results in a green phenotype in some of the cousins. The fate of only one mutagenized R-prime is shown in this figure. See the Discussion and figure 10 for more details.

Figure 10. A Model for Recombination and Deletion of Transposon Tn7 Mutagenized PSA Genes and Chromosomal Alleles. This model details the recombination and deletion processes occuring in step IV of figure 9 which leads to the successful recombination of both the transposon and the crtD lesion into the R. capsulata chromosome. Consistent with data from Southern hybridizations and genetic data on phenotypic expression of mutagenized alleles, one of three mutagenized R-primes recombines with the chromosome and only the recombinant R-prime bearing the wild-type PSA alleles deletes while under streptomycin selection. At this point both the transposon induced lesion and the crtD lesion are expressed, while other mutagenized R-primes are undeleted. Since all R-primes are mutagenized, they do not complement the chromosomal mutations. These mutagenized R-primes will also delete to the parental R-factor when the streptomycin selection is removed and the strains are subcultured. Presumably, maintainence of undeleted R-primes under streptomycin selection is due to a gene-dosage phenomenon whereby bacteria with more copies of Tn7 grow faster in streptomycin medium.

TABLE ONE

Bacterial Strains and Plasmids

Strain	Relevant Properties	Reference/Source
Escherichia coli		
HB101	*pro*⁻, *leu*⁻, *thr*⁻, *lacY*⁻, *hsdM*⁻, *hsdR*⁻, *recA*⁻, Sm^r	S. Cohen
MV12	*thi*⁻, *thr*⁻, *leu*⁻, *trp*⁻, *recA*⁻, *lacY*⁻	N. Panapoulos
NEC0100	MV12, Rf^r	This work
NEC0125	MV12, Nl^r	This work
SK1592	*gal*⁻, *thi*⁻, *hsdM*$^+$, *hsdR*⁻	S. Kushner
SK1593	galactose insensitive derivative of SK1592	N. Panopoulos
SK2267	SK1592, *recA*⁻	S. Kushner
NEC100::Tn7	NEC0100, Tn7 insertion at minute 82	This work
BEC404	HB101 (pRPS404)	Marrs, 1981
US404	NEC0100::Tn7 (pRPS404)	This work
UA#	Library of SK2267 (pRPS404::Tn7)	This work
UAE#	Library of NEC0125 (pRPS404::Tn7)	This work
Rhodopseudomonas capsulata		
SB1003	Rf^r	Yen and Marrs, 1976
Y142	RC⁻, LHI⁻, Sm^r	Drews et al., 1976
UAR#	Library of SB1003 (pRPS404::Tn7)	This work
UAE#ℓ	Cousins* resulting from UAR#	This work
PLASMIDS		
pLM2	RP1 derivative, Km^r, cma$^{\pm}$, Tc^{am}, Am^{am}	Mindch et al., 1976
pBLM2	pLM2 derivative, Km^r, cma$^+$, Tc^{am}, Am^{am}	Marrs, 1981
pRPS404	pBLM2 derivative, Km^r, Tc^{am}, Am^{am}	Marrs, 1981

* bears PSA genes, *crtD233*

TABLE ONE (cont.)

pVCT14	Col E1::Tn7 Sm^r, Sp^r, Tm^r	Lichtenstein & Brenner, 1981
pVCT15	EcoR1 deletion derivative of pVCT14 Sm^r, Sp^r, Tm^r	Lichtenstein & Brenner, 1981
$RP1_{ts}$ Am^s	Km^r, Tc^r, Am^s, rep	Sakanyan, 1978
$RP1_{ts}$::Tn7	$RP1_{ts}Am^s$ bearing Tn7	Sato, 1981

TABLE TWO

Tn7 Insertions in pRPS404

UAE#	Bam H1	Eco R1
3	E	E
15	E	E
18	E	E
19	ND	G
22	E	E
28	E	E
33	ND	D
35	ND	G
37	ND	D
39	D	F
67	F	F
70	C	H
78	C	H
81	C	A
87	F	F
91	C	A
100	E	E
110	NT	E
112	NT	G
115	E	E
118	NT	D
122	NT	G
131	C	A
219	E	E

TABLE THREE

Phenotypes of R. capsulata PSG⁻ Mutants

Strain UAR	Insertion Bam HI	Insertion Eco RI	Color	Polypeptides (kd) 28	24	21	14	12	10	8	Absorption (nm) 590	800	860	Plus
18f	E	E	R	+	+	+	-	+	+	+	+	-	+	
22h	E	E	G	-	-	-	-	-	-	-	-	-	-	
39f	D	F	G	-	-	-	-	-	-	-	-	-	-	
39h	D	F	G	-	-	-	-	-	-	-	-	-	-	
70e	C	H	G	-	-	-	-	-	-	-	-	-	-	665
70f	C	H	G	-	-	-	-	-	-	-	-	-	-	665
70g	C	H	G	-	-	-	-	-	-	-	-	-	-	665
70h	C	H	G	-	-	-	-	-	-	-	-	-	-	665
115e	E	E	R	+	+	+	-	+	+	+	+	L	+	880
115f	E	E	G	+	+	+	-	+	+	+	+	L	+	880
115g	E	E	G	+	+	+	-	+	+	+	+	L	+	880
115h	E	E	G	+	+	+	-	+	+	+	+	L	+	880
219e	E	E	G	-	-	-	-	-	-	-	-	-	-	

Figure 1

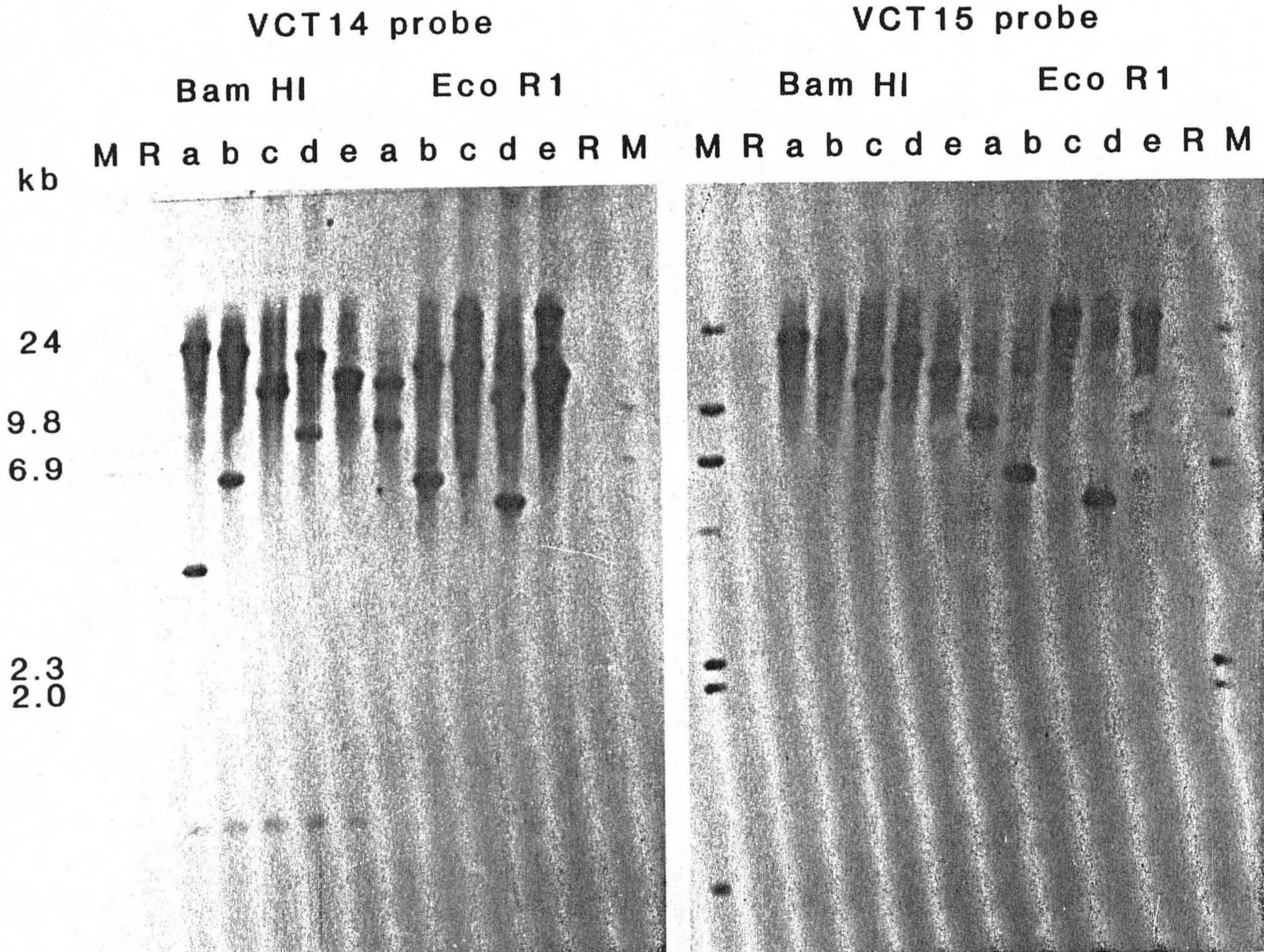
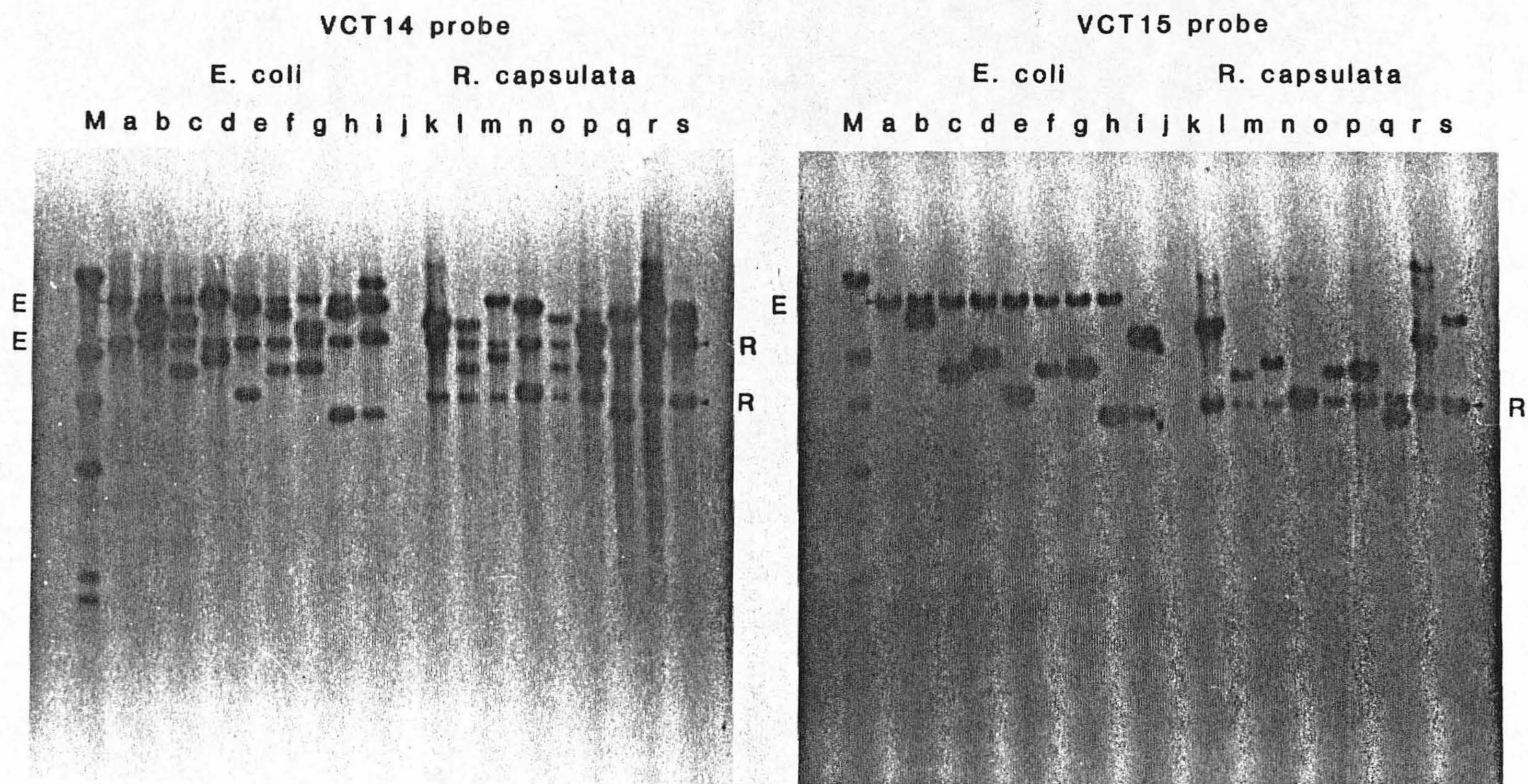


Figure 2



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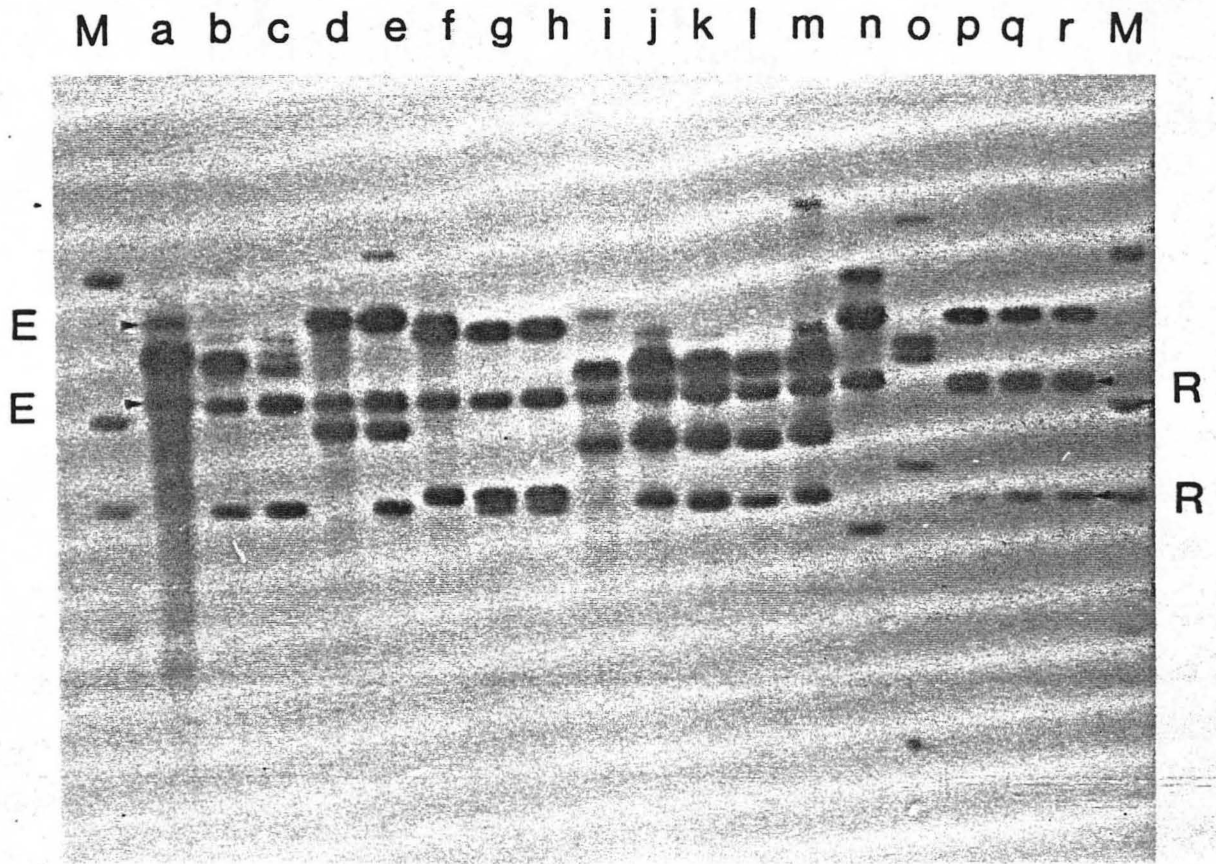

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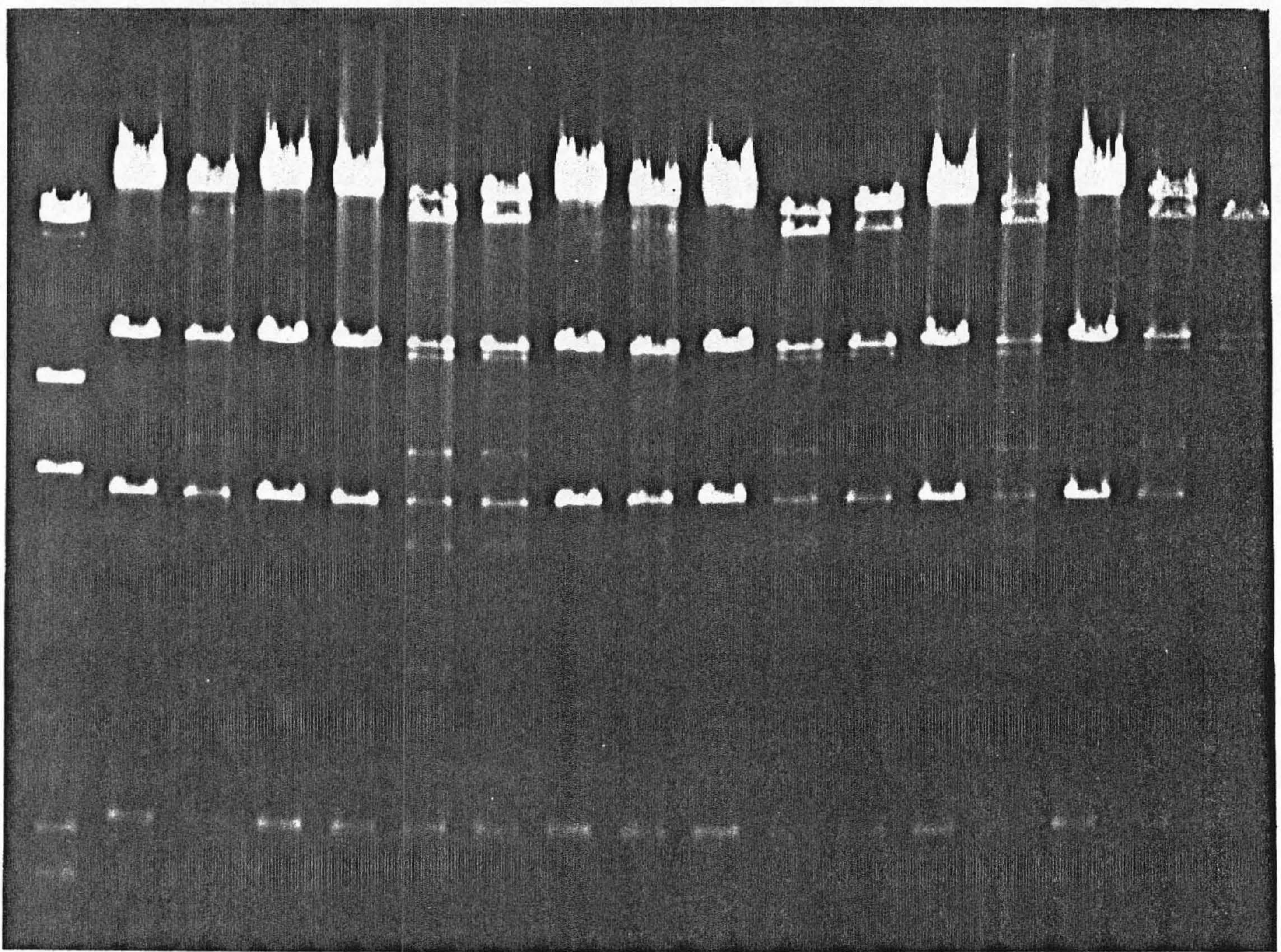

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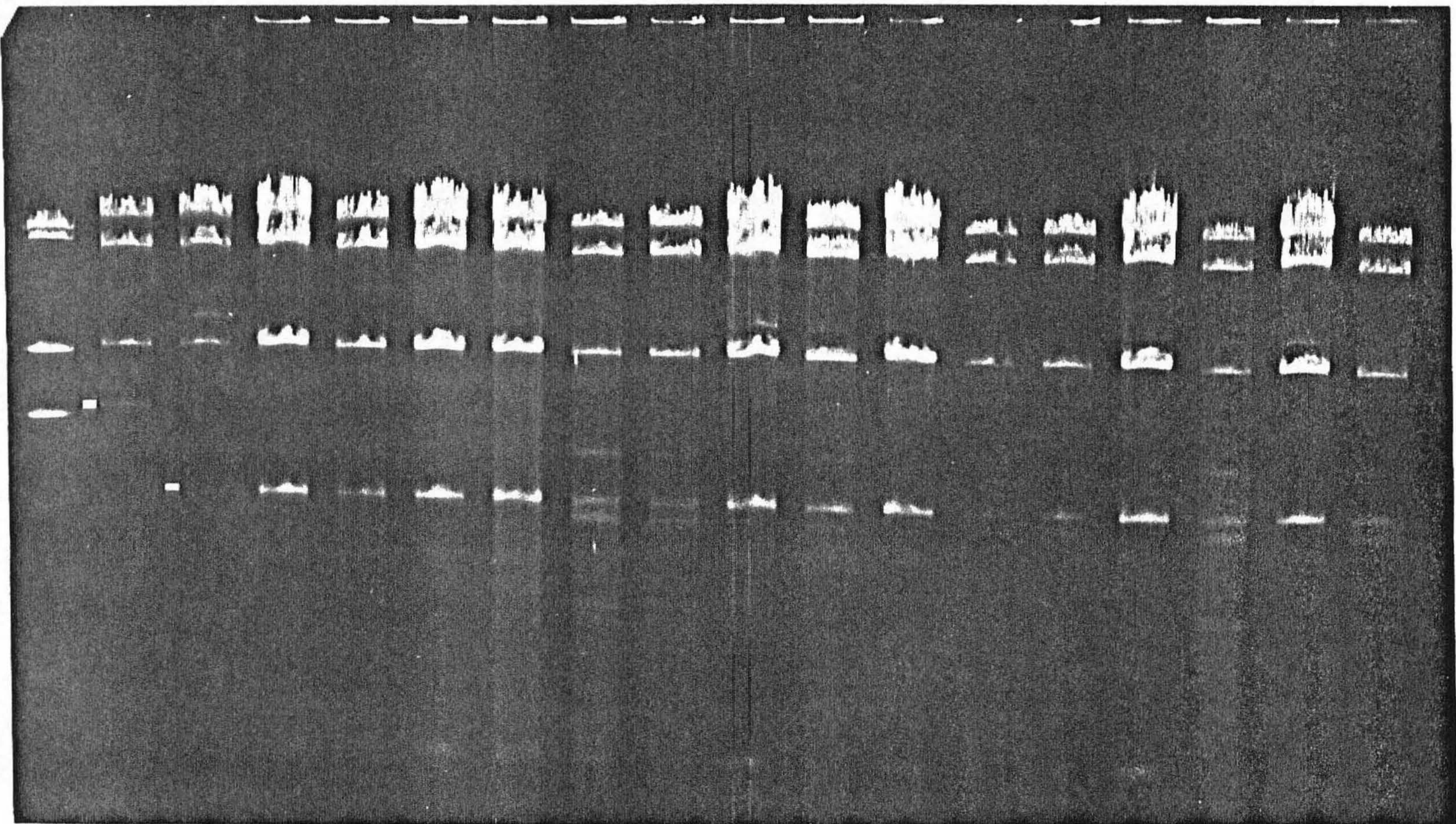

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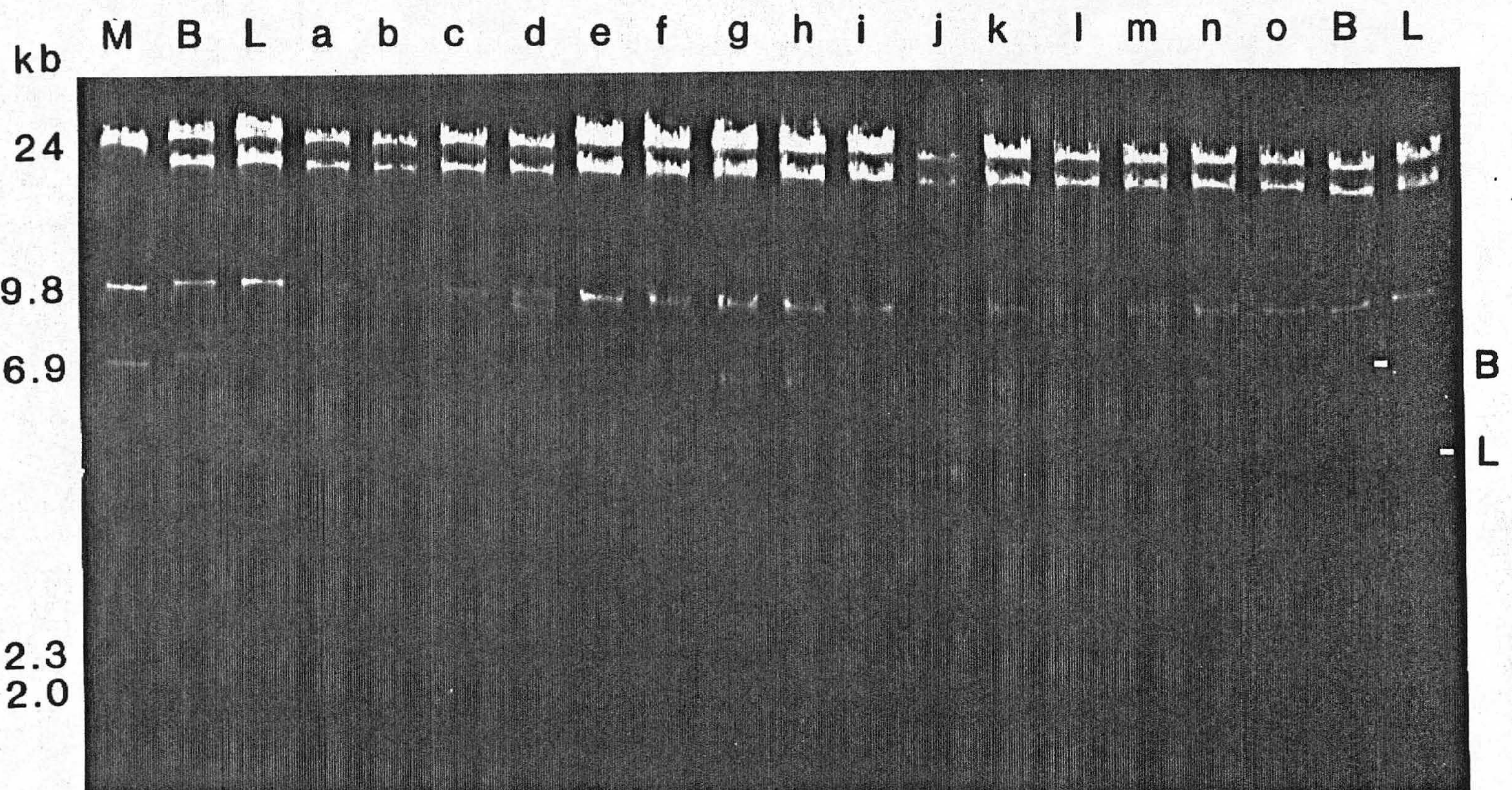


Figure 7

R prime probe

Bam H1 Eco RI

M a b c d e f g h i M a b c d e f g h i

A B

C

D E

F G,H

I

J

K

A,B

C E

F

G

H

I

J,K,L

M

Tn7 probe

Bam H1 Eco RI

kb M a b c d e f g h i M a b c d e f g h i

24

9.8

6.9

4.4

2.3

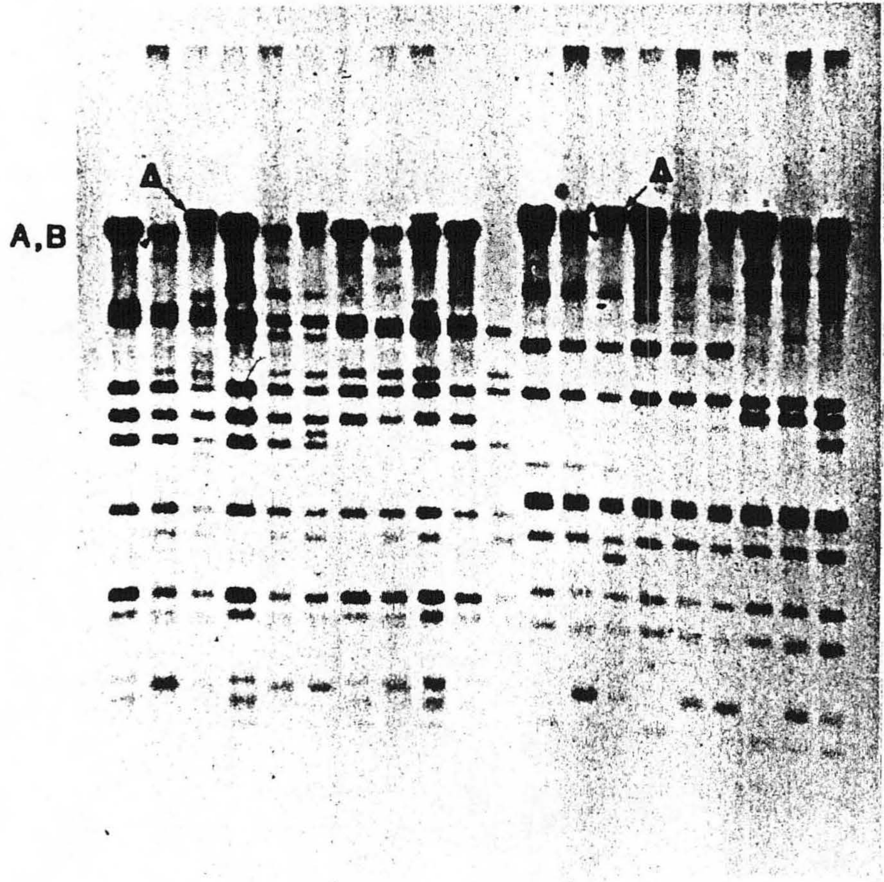
2.0

Figure 8

R-prime probe

Eco R1 **Bam HI**

a b c d e f g h i j k a b c d e f g h i



R-factor probe

Eco R1 **Bam HI**

a b c d e f g h i j k a b c d e f g h i

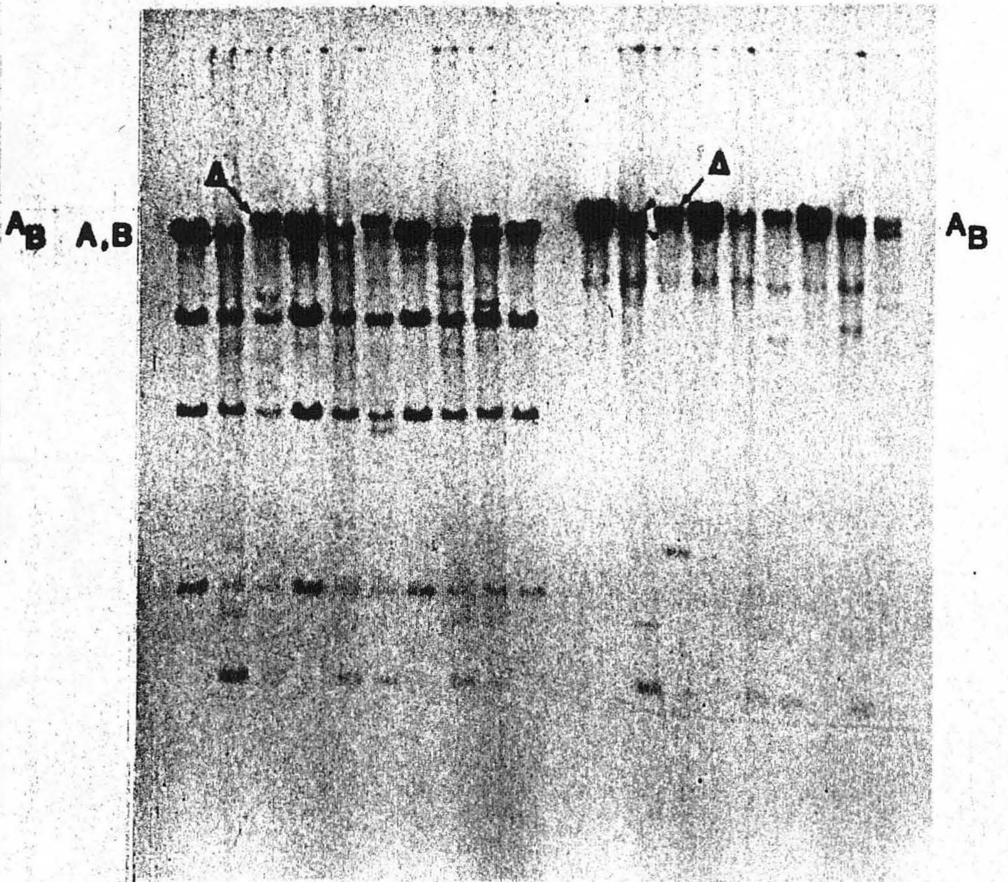
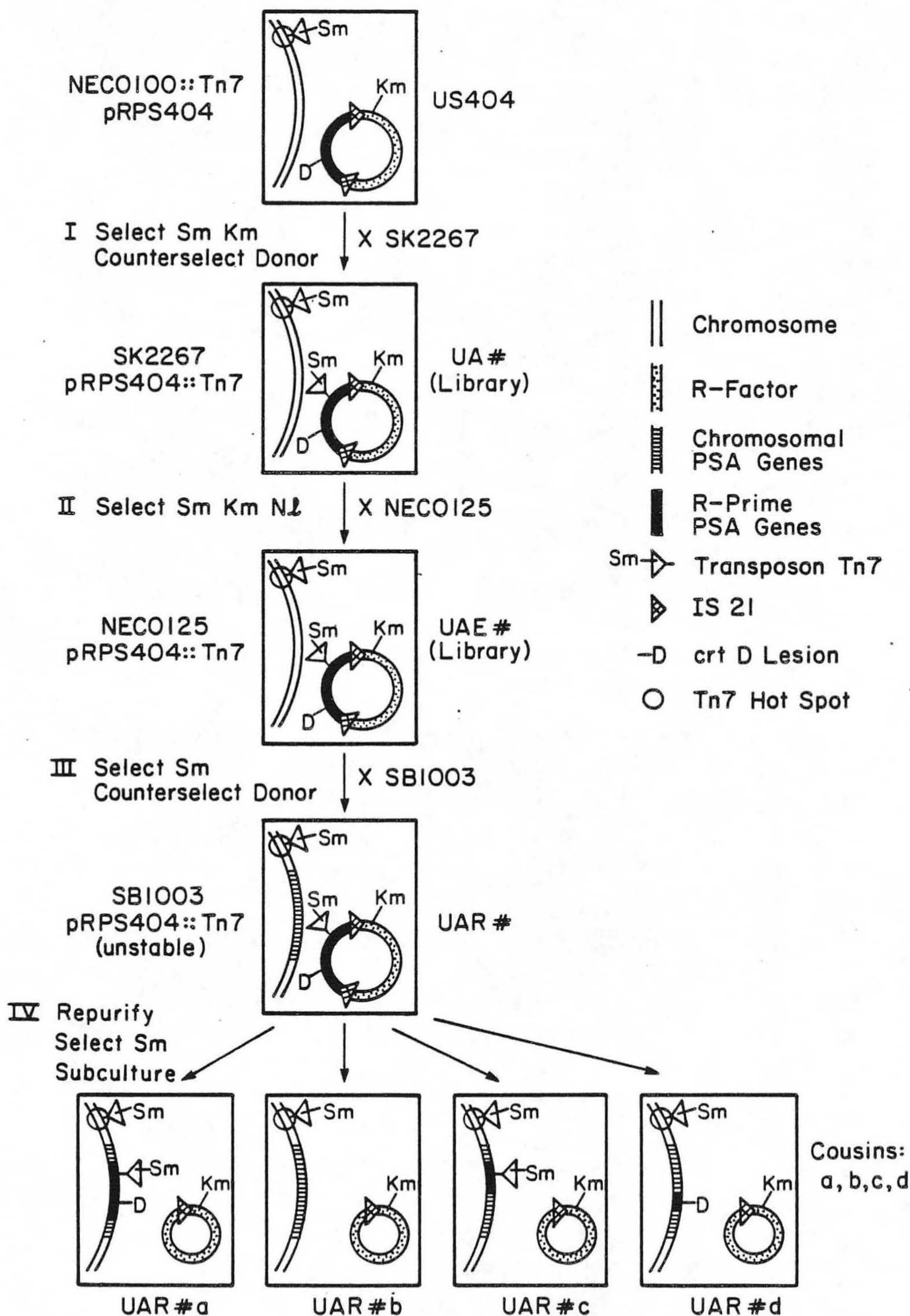


Figure 9

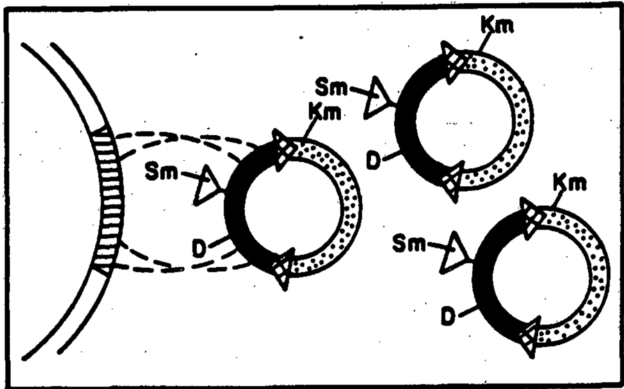
CLONAL SITE-DIRECTED TRANSPOSON MUTAGENESIS



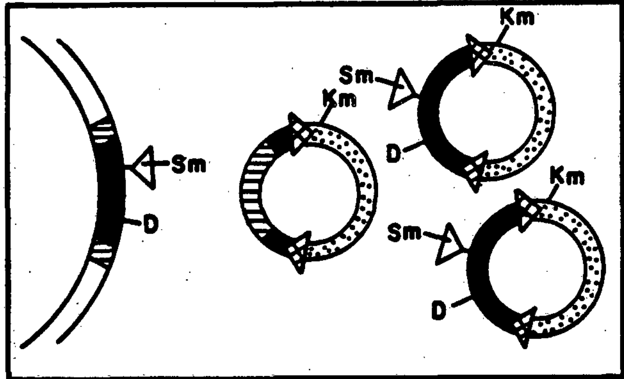
XBL 823-8829

Figure 10

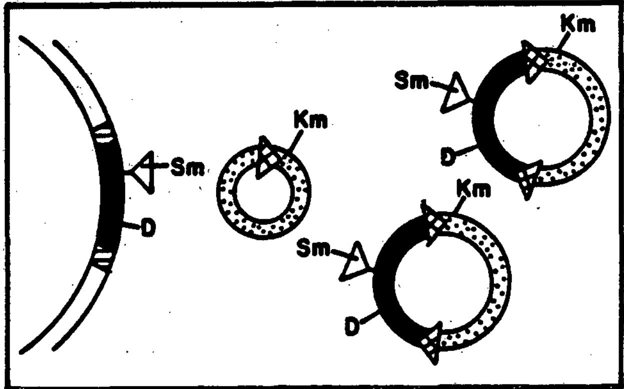
Recombination and Deletion



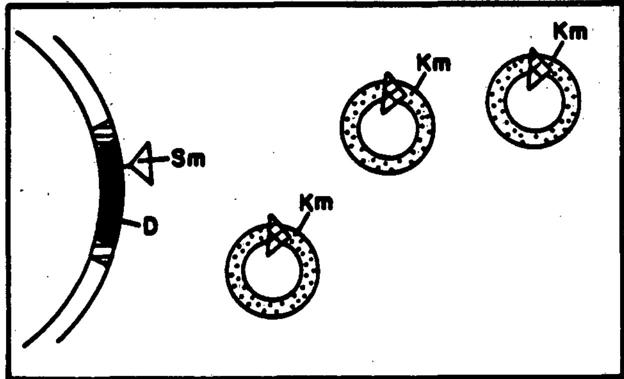
I. Recombination — Select Sm



II. Wild-Type Alleles Delete — Select Sm



III. All Primes Delete — No Selection



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