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Genetics

Homologous recombination in mammalian cells mediates formation of a functional gene from two overlapping gene fragments

(mitotic recombination/gene introduction/gene fusion)

BRUNO ROBERT DE SAINT VINCENT* AND GEOFFREY M. WAHL†

Tumor Virology Laboratory, The Salk Institute, La Jolla, California 92037

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ABSTRACT **Chinese hamster cells with a lesion in the CAD gene (cell line Urd⁻A) require exogenous uridine to survive. Uridine prototrophs could be isolated after introducing two recombinant plasmids containing overlapping fragments of a cloned Syrian hamster CAD gene. In contrast, no uridine prototrophs were obtained after introducing a plasmid containing only one of the two overlapping fragments. DNA restriction analysis showed that the prototrophic transformants contain a functional CAD gene which was formed by a recombination event in the overlapping region of the two clones. Most of the recombination events involved homologous exchanges, and some of them apparently were reciprocal. *In situ* hybridization analysis revealed that the donated sequences were integrated at a single chromosomal site which was different in each transformant. These results demonstrate the existence of a recombination system(s) in mammalian cells that can catalyze homologous exchanges. Recombination between donated sequences is a means by which this system can be characterized and also utilized for the production of novel gene fusions.**

Various pieces of evidence indicate that recombination between DNA sequences can occur in mitotically dividing eukaryotic cells. For example, Stern (1) invoked mitotic recombination to explain the formation of twin spots in *Drosophila* somatic cells, and genetic experiments have demonstrated that mitotic recombination occurs in fungi (2–5), slime molds (6), and plants (7). Recent experiments have shown that mitotically dividing yeast recombine introduced exogenous DNA molecules in a sequence-specific manner, providing an effective and useful tool for directing purified genes into specific regions of the yeast genome (8).

Cytogenetic and biochemical studies of mitotically dividing mammalian cells indicate they also catalyze recombination events. It has been known for 25 years that exchanges between sister chromatids is a frequent event in diverse mammalian species (9, 10) and that such exchanges involve interstrand transfers of DNA (11, 12). Cytogenetic banding techniques have shown that unions between nonsister chromatids to form "quadri-radial" structures (13) also involve exchanges of chromosomal material (14).

The introduction of DNA molecules of defined structure into mammalian cells provides a powerful tool for analyzing the substrate requirements and enzymatic steps that mediate recombination in animal cells. It has been possible to use this strategy to show that overlapping fragments of simian virus 40 or adenovirus introduced into monkey (15, 16) or human (17) cells are recombined in the homologous region to yield hybrid viruses. In contrast to such apparently homologous events, analysis of the fates of DNA molecules introduced by various techniques indicates that mammalian cells can also join DNA sequences in regions of little (18–20) or no homology (19).

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In this report, we describe a model system for analyzing the capacity of mammalian cells to catalyze homologous exchange events. We show that two chimeric plasmids containing overlapping regions of the CAD gene can be introduced into animal cells and recombined sufficiently precisely in the overlapping region to form a functional CAD gene. Because the recombination substrates consist only of genomic and plasmid sequences, recombination between these molecules is strictly dependent upon the action of the host cell recombination machinery.

METHODS

Plasmid Vectors. The plasmid vectors pDH24 and pDS4 were used in these experiments [kindly provided by D. Hare and J. Sadler (University of Colorado) and D. Stocker and D. Helinski (University of California at San Diego), respectively]. pDH24 was prepared by cleaving pBR322 at the *Eco*RI site, removing the protruding nucleotides, and ligating the blunted ends to *Xba* I linker fragments (C-T-C-T-A-G-A-G, Collaborative Research, Waltham, MA) which oligomerized during the ligation reaction. Linker oligomerization created at least one *Sst* I site (G-A-G-C-T-C) between several *Xba* I sites. pDS4 is a derivative of pACYC184 and is compatible with pBR322 (21).

Construction of Plasmids with Overlapping Fragments of the CAD Gene. The cosmid cCAD1 (22) was digested to completion with either *Eco*RI or *Sst* I and the restriction fragments were fractionated by electrophoresis through a 0.5% SeaPlaque (Marine Colloids, Rockland, ME) agarose gel. A 19-kilobase (kb) *Eco*RI fragment which contains most of the coding unit and 1.5 kb of sequences downstream from the 3′ end of the CAD mRNA and a 13-kb *Sst* I fragment which contains the remainder of the coding unit and approximately 5 kb of sequences upstream from the 5′ end of the CAD mRNA (ref. 23; unpublished data) were excised from the gel. The fragments were ligated to *Eco*RI- or *Sst* I-cleaved and alkaline phosphatase-treated pDS4 or pDH24, respectively, in the presence of the agarose (24). Recombinants containing either fragment in the two possible orientations were isolated for further study.

RESULTS

Introduction of Overlapping CAD Fragments into CAD-Deficient Cells. The mutant Chinese hamster ovary cell line Urd⁻A has a lesion in the CAD gene. Therefore, these cells cannot synthesize uridine *de novo* and consequently require an exogenous supply of uridine for growth (25). In previous experiments, we showed that the auxotrophy of the Urd⁻A cells could be stably reversed by fusing them with *Escherichia coli*

Abbreviations: CAD, multifunctional enzyme which catalyzes the first three steps of uridine biosynthesis in higher eukaryotes (this enzyme contains carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, and dihydro-orotase activities); kb, kilobase(s).

* Present address: Institut Pasteur, 75724 Paris, France.
† To whom reprint requests should be addressed.

protoplasts harboring a cosmid containing a functional Syrian hamster CAD gene (22). We reasoned, therefore, that selecting for uridine prototrophs after introducing two overlapping and nonfunctional CAD gene fragments would provide an assay for recombination in Urd$^-$A cells.

Table 1 shows that uridine prototrophs could be obtained by fusing Urd$^-$A cells with protoplasts of recA$^-$ *E. coli* (either strain HB101 or DH1) containing two plasmids with overlapping regions of the CAD gene (e.g., Sst-7 and RI-15 or Sst-4 and RI-22 as described in Fig. 1). The region of overlap between the CAD gene fragments is 3 kb, it contains no repetitive sequences, and it has at least eight small exons (100–200 base pairs long) (23, 27). The frequency of obtaining prototrophs was approximately 10^{-5} to 10^{-6} (Table 1). There was little difference in the frequency of transformants when the CAD gene fragments were in opposite orientations in the plasmids. Uridine prototrophs were also obtained at a comparable frequency when the Urd$^-$A cells were fused with a mixture consisting of equal numbers of protoplasts carrying each CAD gene subclone separately (experiment 3, Table 1). On the other hand, no prototrophs were obtained from $>10^6$ Urd$^-$A cells in three independent experiments when freshly subcloned populations of Urd$^-$A cells were fused with bacteria containing only one of the CAD gene subclones.

These experiments demonstrate that both of the overlapping fragments are required to relieve the CAD deficiency of the Urd$^-$A cells. Because equal frequencies of prototrophs were obtained when the overlapping fragments were in the same or different bacteria and the bacteria used were recombination deficient, the above experiments suggest that the fragments were recombined to form functional CAD genes in Urd$^-$A cells. This idea is further supported by our inability, using DNA blot hybridization (data not shown), to detect the expected recombination products in plasmid DNA isolated from either recA$^+$ or recA$^-$ bacteria harboring both overlapping plasmids. If these plasmids recombined in *E. coli*, the frequency was too low to account for the number of uridine prototrophs we observed.

Table 1. Rescue of CAD-deficient cells after introduction of overlapping CAD gene fragments

Plasmids	Wells with colonies, total of wells no./no.	Frequency, $\times 10^6$
	Exp. 1	
Sst-4, RI-15	3/6	6.93
Sst-7, RI-15	4/6	11
Sst-7, RI-22	1/6	1.83
Sst-4, RI-22	3/6	6.93
Sst-4	0/6	—
RI-15	0/6	—
None	0/6	—
	Exp. 2	
Sst-4, RI-15	3/10	3.57
Sst-7, RI-15	4/10	5.1
Sst-7	0/6	—
RI-15	0/6	—
None	0/4	—
	Exp. 3	
Sst-4	0/6	—
RI-22	0/6	—
(Sst-4) (RI-22)	6/12	6.93
None	0/6	—

CAD-deficient Urd$^-$A cells grown in six-well, 35-mm dishes (1×10^5 cells per dish) were fused with protoplasts made from bacteria (strain HB101, Exps. 1 and 3; DH1, Exp. 2) harboring the indicated CAD gene subclone(s) (see Fig. 1). The number of uridine prototrophs were determined after 2–3 weeks of growth in uridine-free medium (usually 1–5 colonies per 10^5 cells). The frequency of obtaining prototrophs was estimated from the proportion of wells having no colonies (26). In all experiments, except as indicated by the parentheses in Exp. 3, both CAD subclones were introduced from the same bacterium.

Urd$^-$A Cells Recombine the Overlapping CAD Sequences to Form a Functional CAD Gene. Direct evidence that recombination between the overlapping sequences of the CAD gene subclones mediated the formation of active CAD genes in the prototrophic transformants was obtained by analyzing the restriction patterns of the donated CAD sequences. One useful enzyme for these studies is *Xba* I. The pattern of *Xba* I restriction fragments expected to result from recombination between plasmids Sst-7 and RI-15 is shown in Fig. 1. The 13-kb *Sst* I fragment containing the 5′ end of the CAD gene was inserted into a derivative of pBR322 to generate plasmid Sst-7. Because the insert in Sst-7 contains *Xba* I sites at each end (provided by the *Xba* I linkers of the vector) but does not contain internal *Xba* I sites, it will be excised intact after *Xba* I digestion. The 19-kb *Eco*RI fragment containing the 3′ end of the CAD gene was inserted into vector pDS4 to generate plasmid RI-15. RI-15 has several *Xba* I sites, of which one is 2.8 kb downstream from the *Sst* I site that marks the 3′ boundary of the overlap region (Fig. 1). Another *Xba* I site is located in the adjacent cloning vector sequences approximately 0.6 kb from the *Eco*RI site (the junction between plasmid and insert sequences).

When Sst-7 and RI-15 DNA were digested with *Xba* I, fractionated on agarose gels, transferred to nitrocellulose, and hybridized with a probe that spans part of the overlapping region of these plasmids (referred to as probe 104), a 13-kb and a 6.6-kb fragment, respectively, were seen (Fig. 2*A*, lanes K and J). However, if a recombination event occurs within the overlap region of plasmids Sst-7 and RI-15, then *Xba* I cleavage of the recombined molecules will yield a fragment 15.8 kb long which hybridizes with probe 104. If the recombination is reciprocal, *Xba* I digestion will produce an approximately 3.8-kb fragment which extends from the *Xba* I–*Sst* I site at the 3′-proximal terminus of the insert in Sst-7 to the *Xba* I site in the vector sequences of RI-15 (Fig. 1).

Fig. 2 shows the hybridization patterns obtained from approximately equal amounts of *Xba* I-cleaved DNA isolated from the indicated transformants. All of these transformants have the expected 15.8-kb band (indicated by an arrow), and the intensity of this band was different in independent transformants. There also was a band at 3.8 kb in one of the two transformants made with RI-15 and Sst-7 and in the one transformant made with RI-15 and Sst-4 (Fig. 2, lanes C and E). The intensity of the 3.8-kb band reflects that of the 15.8-kb band.

The DNA restriction patterns of the transformants obtained by introducing plasmids Sst-4 and RI-22 also contained the expected 15.8-kb fragment. In these recombinants, a reciprocal exchange is indicated by *Xba* I fragments approximately 6.2 or 5.0 kb long depending upon whether the recombination event took place in the left- or right-hand insert of RI-22 (Fig. 1). The 6.2-kb fragment cannot be used to indicate a reciprocal exchange because the endogenous CAD sequences exhibit a band of hybridization of approximately this size. However, a 5.0-kb fragment was seen in two of the three recombinants studied, and the intensity of this band reflected that of the 15.8-kb fragment in the respective lines (Fig. 2, lanes B and G).

If the observed 15.8-kb *Xba* I fragment does represent recombined functional CAD genes and if each recombined gene is expressed equally, then the intensity of the 15.8-kb fragment should reflect the CAD specific activity of the transformants. In most cases the CAD specific activity (measured by determining the aspartate carbamoyltransferase activity) did reflect

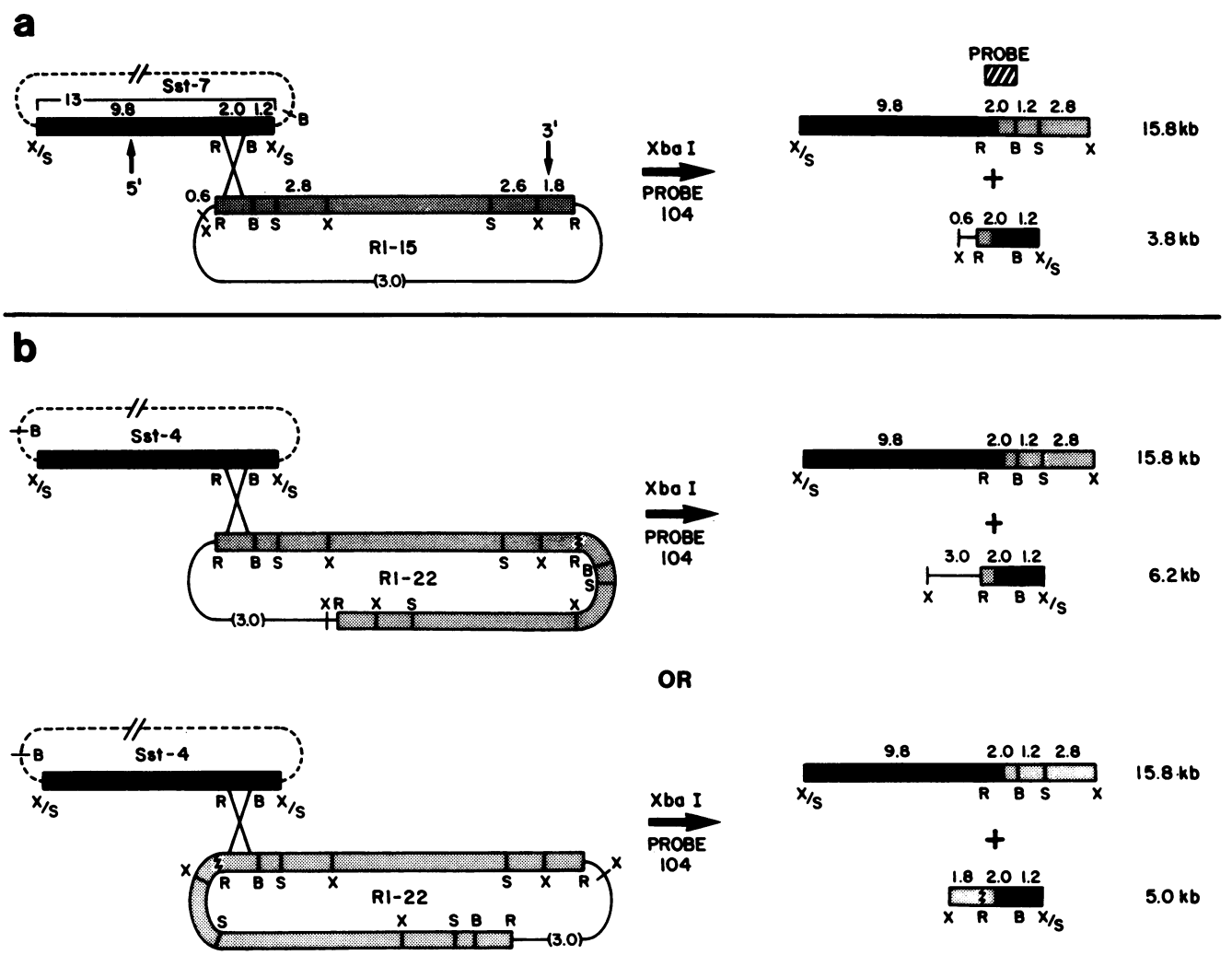

FIG. 1. Simplified restriction map of the CAD gene subclones and the expected products of their recombination. The restriction sites relevant to the studies in this paper are shown: B, *Bam*HI; R, *Eco*RI; S, *Sst* I; X, *Xba* I. The sizes of the fragments between the indicated restriction sites are shown in kb. The approximate 5′ and 3′ boundaries of the CAD encoding sequence are indicated by vertical arrows. The probe used for all hybridization experiments was CAD fragment 104 which extends from the *Bam*HI site to the *Eco*RI site of the overlap region as indicated in the right side of the figure. (*a*) Recombination between plasmids Sst-7 and RI-15. (*b*) The two recombination events that could occur between the overlapping regions of plasmids Sst-4 and RI-22. The plasmid cloning vectors are identical in size in RI-15 and RI-22 and appear larger in the top drawing only to preserve the alignment of the CAD inserts in the three diagrams shown.

the intensity of the hybridization signal of the 15.8-kb fragment (Table 2). One apparent difference can be seen for transformant CA1 (Fig. 2, lane G) which has about one-fourth the CAD activity expected for a 15.8-kb band of the observed intensity. This difference could reflect lower expression of the recombined genes due to either the site of chromosomal insertion or some imprecise recombination events in the overlapping region. Due to their various CAD levels, the transformants also exhibit different levels of resistance to the carbamoyltransferase inhibitor PALA (Table 2).

Other Homologous Recombination Events in the Donated CAD Sequences. The restriction digest patterns in Fig. 2 show that the most intense hybridization in each transformant occurred to fragments that comigrated with those of the parental plasmids. Analysis of DNA from those transformants with other restriction enzymes (e.g., *Sst* I) and with probes spanning the CAD and vector sequences revealed discrete, intense bands expected from the restriction maps of the donated sequences (data not shown). Introduction of a functional CAD gene in a cosmid vector also yielded the expected restriction fragment (Fig. 2, lane H). The data are consistent with the presence of an oligomer of CAD sequences formed by homologous recombination.

Donated CAD Sequences Are Integrated at Single Chromosomal Sites in Each Transformant. The transformants isolated thus far retain the capacity to grow under selective conditions after passage for more than 60 generations without selection (data not shown). The stable prototrophic phenotype of the transformants suggested that the donated DNA was integrated into the host chromosomes. We tested this idea directly by *in situ* hybridization of the metaphase chromosomes of transformants with 20–100 copies of the donated sequences (29). The probe that was used, p102 (23), is homologous with both recombination substrates because it contains CAD sequences at the 3′ end of the insert in plasmids RI-15 and RI-22, and its vector, pBR322, is present in plasmids Sst-4 and Sst-7. Even in lines with as many as 100 CAD sequences which have undergone extensive recombination, most if not all of the donated sequences resided in a single chromosomal location (Fig. 3). However, a different chromosome contained the donated sequences in each transformant.

DISCUSSION

We have examined the capacity of CAD-deficient, uridine-dependent CHO cells to recombine overlapping, nonfunctional

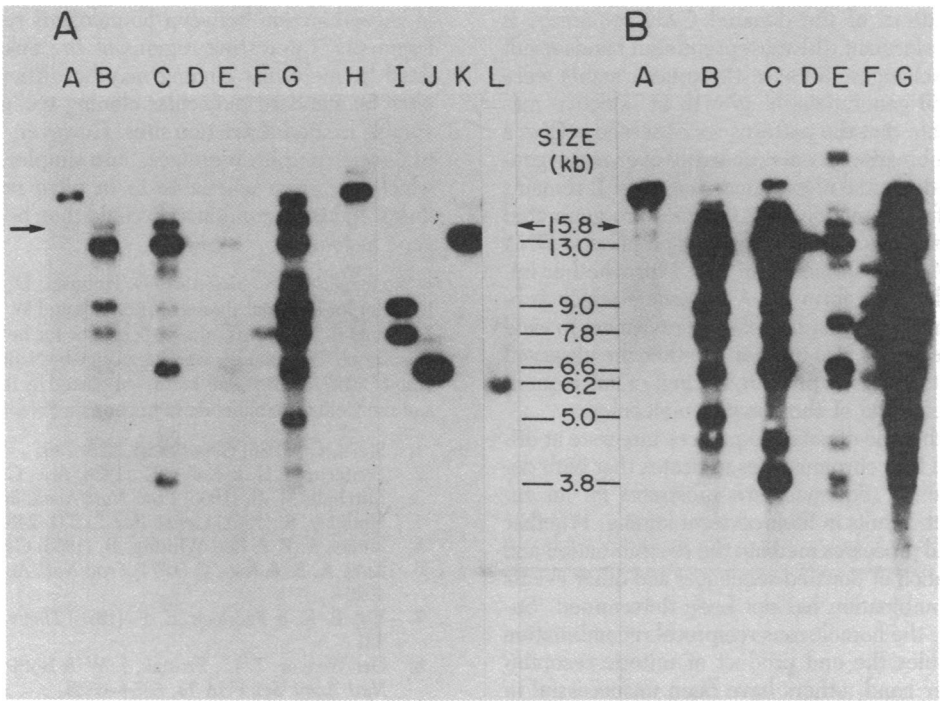

FIG. 2. Restriction analysis of chromosomal DNA isolated from prototrophic transformants. Clones of prototrophic transformants were picked and grown into mass culture for the isolation of high molecular weight DNA. DNA (approximately 10 μg unless indicated otherwise) from the indicated cell lines and plasmids (≈1 ng) was digested to completion with a 5-fold excess of *Xba* I, fractionated on a 0.7% agarose gel, transferred to nitrocellulose (Schleicher & Schuell, BA 83), and hybridized as described (28). The probe was CAD fragment 104 (23). The exposures were 6 hr for lanes A–K in *A* and 23 hr for lane L in *A* and all lanes in *B*. The lanes contained DNA from the following sources (the plasmids introduced into the indicated transformants are in parentheses): A, 165-28 (a PALA-resistant SV-28 cell line with 200–300 CAD genes, 1 μg); B, R21 (RI-22, Sst-4); C, R17 (RI-15, Sst-7); D, R15 (RI-15, Sst-7; less DNA was loaded in this lane as indicated by the lower hybridization to the 6.2-kb band of Urd⁻A CAD sequences); E, R12 (RI-15, Sst-7); F, DA1 (RI-22, Sst-7); G, CA1 (RI-22, Sst-4); H, K1-PALA3 (a transformant made by introducing a functional CAD gene cloned in a cosmid into CHO-K1 cells); I, plasmid RI-22; J, plasmid RI-15; K, plasmid Sst-7; L, CHO-K1. The *Xba* I sites used in these studies apparently were not methylated in the bacterial strains we used because the restriction maps derived from the plasmids were consistent with those obtained from Syrian hamster genomic DNA. The fragment obtained by *Xba* I digestion of 165-28 or K1-PALA3 DNA (lanes A and H) does not have the same mobility as a recombinant CAD gene because the 5′-proximal *Xba* I site of plasmid Sst-7 does not exist in a wild-type CAD gene and was introduced into the gene via the *Xba* I–*Sst* I linker fragment of the cloning vector.

fragments of a Syrian hamster CAD gene to form a functional CAD gene. Such an event is detected by the growth of uridine-independent colonies. The region of overlap is punctuated by at least eight small exons separated by introns that are 50–150 base pairs long and contain no repeated DNA sequences (23, 27). Formation of a functional CAD gene by recombination within the overlap region requires the recombination event to preserve the complex RNA splicing patterns in this portion of the gene and to be sufficiently precise to avoid creating lesions that result in significant amino acid substitutions, deletions, or frame changes. Because the recombination substrates contain only bacterial plasmid and CAD sequences, the formation of a functional CAD gene must be accomplished by host recombination functions. This distinguishes our approach from similar ones measuring recombination between viral sequences in which cases virally encoded recombination factors may be involved (15–17).

Table 2. CAD levels in recombinants

Cell line	Lane in Fig. 2	CAD level	PALA LD_{50}, μM
CHO-K1	L	0.27	30
R21	B	0.50	100
R17	C	0.75	150
R15	D	0.050	2
R12	E	0.20	30
DA1	F	0.038	1
CA1	G	0.69	100

CAD levels were estimated by measuring aspartate carbamoyltransferase specific activity (μmol/mg of protein per 30 min) as described (23). LD_{50} is the PALA concentration that decreases the relative plating efficiency by 50%. Transformants R15 and DA1 have approximately one or two functional recombinant CAD genes because the enzyme specific activity in these lines was approximately equal to that of BHK 21 cells [the parents of the cells used for cloning the CAD gene (22)] and the signals generated by the recombinant and endogenous CHO CAD genes were approximately equal (compare, in Fig. 2*B*, lanes D and F, the intensities of the 15.8-kb band and the 6.2-kb band which is derived from the endogenous CAD sequences).

Our results demonstrate that the Urd⁻A cells that we used contain the enzymatic machinery required to recombine donated DNA sequences in a region of homology. In some cases, the recombination event appears to be reciprocal. Although the

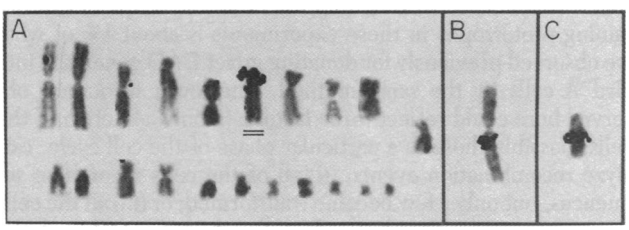

FIG. 3. Chromosomal localization of the recombined CAD genes. Metaphase spreads were prepared from the indicated Colcemid-arrested cell lines and hybridized with the ^{3}H-labeled CAD-specific probe p102 as described (29). (*A*) Typical pattern of hybridization in transformant CA1. The chromosome indicated by the double underlining was the only one exhibiting reproducible hybridization in the 10 metaphase spreads that were examined. (*B* and *C*) The only chromosomes in transformants R21 and R17 that hybridized reproducibly (10 metaphases examined per transformant).

restriction digest pattern of the donated CAD sequences is complex in each transformant, the most prominent bands result from homologous exchanges. Because the transformants were analyzed after 30–60 generations of growth in selective medium, it is conceivable that the patterns we observed reflect a summation of recombination events occurring over many generations rather than the initial recombination events. It remains to be determined if the functional CAD genes are generated by recombination between circular or supercoiled plasmid DNA molecules (as indicated for simplicity in Fig. 1) or whether initial recombinational events form DNA oligomers which subsequently undergo intramolecular homologous exchanges to yield the observed products. It is also unclear whether the observed recombination events occurred prior to, during, or subsequent to chromosomal integration of the donated molecules.

Our observation that the donated sequences integrate at different regions of the host chromosomes indicates that both donated and chromosomal sequences are substrates for an enzymatic process which results in their covalent joining. Whether the same biochemical processes mediate the recombination and chromosomal integration of donated sequences and other events such as mitotic recombination has not been determined. Superficially, however, the homologous reciprocal recombination we observed resembles the end product of mitotic recombination. On the other hand, others have been unsuccessful in their attempts to find recombination between X-linked (30, 31) or autosomal sequences (32) in a cell line derived from the same parent as used in the present studies.

It is conceivable that donated DNA molecules are better substrates for recombination than are chromosomal sequences in mitotically dividing cells because they have nicks or gaps which serve as nucleating sites for recombination events. It is interesting in this regard that the high spontaneous frequency of meiotic recombination has been correlated with the occurrence of single-stranded breaks (33–35) and that persistent strand breaks are thought to lead to recombination (36). Perhaps the comparably low frequency of mitotic recombination reflects the low probability of such lesions persisting in the DNA of vegetative cells (37). It is also possible that introducing free or damaged DNA molecules induces a repair or recombination system. Another possibility is that the recombination frequency of CHO cells is dependent on the copy number of the target sequences. This threshhold may be overcome easily by the numerous molecules introduced by protoplast fusion. Lastly, it is conceivable that the genetic experiments cited above used markers that are located in regions of low recombinational activity (see refs. 30 and 32 for further discussion of this point).

Our experiments do not address the question of whether all cells in a population can recombine DNA sequences because our data reflect both the frequencies of recombination and transformation. However, it appears that the frequency of obtaining prototrophs in these experiments is about 1% of what we observed previously for donating intact CAD genes (22) into $Urd^{-}A$ cells by the same method. The lower frequency observed here could reflect three factors: (*i*) only a fraction of the cells, possibly those in a particular phase of the cell cycle, catalyze recombination events; (*ii*) all of the cells recombine sequences, but only a few become transformed; or (*iii*) all the cells recombine sequences, but only a few have recombined the overlapping fragments in the precise fashion required to form a functional CAD gene. Because only 2 of the 20–100 plasmids introduced by protoplast fusion have to recombine to form a functional CAD gene in order to allow for the cell to survive the selection imposed (22, 25), we think that imprecise recombination is not likely to be a major factor leading to a lower recovery of prototrophs.

A practical application also derives from our demonstration of recombination between homologous regions of CAD gene fragments. Interesting regions of the eukaryotic genome isolated by molecular cloning may be difficult to manipulate *in vitro* by standard molecular cloning techniques, due to unfavorably located restriction sites. However, it should be possible to dissect complex sequences into simpler, overlapping pieces which are more amenable to *in vitro* recombination procedures. *In vivo* recombination could then be used to create novel gene fusions.

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