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Segregation and Rearrangement of Coamplified Genes in Different Lineages of Mutant Cells That Overproduce Adenylate Deaminase

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Four genes encoding proteins designated as W, X, Y1, and Y2 were found previously to be amplified at different levels in a Chinese hamster fibroblast mutant line selected for overproduction of adenylate deaminase. To gain information on the molecular mechanisms responsible, we studied the levels of amplification and the structures of these four genes in several lineages of mutant cells with comparable activities of adenylate deaminase, the selected enzyme. Only the W gene was amplified in all the lines. In one line, the X, Y1, and Y2 genes were coamplified, while in others either the Y1 gene or the pair X and Y2 were coamplified. The results were consistent with linkage of all the genes—in a particular order—in an amplifiable sequence with variable endpoints. Novel joints with a nonrandom distribution were observed. We frequently detected rearranged copies of the W gene, but very few novel joints were present in the other three genes in the six highly amplified lines examined. Some of the novel joints in gene W were highly amplified; they were generated by reamplification of a rearrangement that appeared at an early selection step. In some lines, reamplification was accompanied by deletion or mass correction of preexisting units. We discuss mechanisms which might account for these observations.

We have shown previously (4) that unstable mutants overproducing adenylate deaminase (AMPD) can be isolated from Chinese hamster fibroblast line GMA32 by supplementing the growth medium with adenine, azaserine, and the AMPD inhibitor coformycin. Stepwise increases in the concentration of coformycin allow the recovery of mutants which accumulate increasing amounts of the target enzyme. Proteins other than AMPD also accumulate during these selections, and their levels return to those of wild-type cells in revertants with wild-type levels of AMPD activity. Plasmid cDNA probes specific for genes encoding four such proteins—designated W, X, Y1, and Y2—were isolated and used to show that the four genes are amplified at different levels in the AMPD-overproducing mutants studied in the previous experiments (5). We report here an analysis of the abundance, structure, and expression of these four genes in a variety of clones with comparable levels of AMPD activity, derived independently through multiple steps of selection. We again observed that the four genes were not coamplified to the same extent within the same line. Moreover, the pattern of amplification differed from clone to clone. In some cases, striking differences between the amplification and transcription levels of a gene were detected; they were shown to result from genetic rearrangements. The rearrangements were not randomly distributed along the amplified units, as judged from their preferential location in one of these genes. We discuss mechanisms able to generate the coamplification pattern observed in these clones.

MATERIALS AND METHODS

Cell lines and mutant selection. The GMA32 line of Chinese hamster fibroblasts, conditions for its growth, and the details of stepwise selection to yield coformycin-resistant mutants have been reported previously (3, 4).

Preparation of nucleic acids and hybridization experiments. Total poly(A)$^+$ mRNAs and genomic DNAs were prepared as described previously (5). The isolation of cDNA probes H3, H4, H6, and H11 for genes W, X, Y2, and Y1, respectively, and the conditions used in Northern and dot blot experiments have also been described previously (5). Transfers of genomic DNA were done as described by Southern (9).

Restriction analysis of genomic DNAs. The restriction enzymes *Pst*I, *Eco*RI, *Sac*I, *Bam*HI, and *Pvu*II were obtained from Boehringer Mannheim Biochemicals, and DNAs were digested as directed by the supplier.

RESULTS

Isolation of three families of cell lines which overproduce AMPD. Three clones, HC4, HC5, and HC6, were isolated from the GMA32 line in selective medium containing 0.5 μg of coformycin per ml (step I mutants). Although they were recovered from different ethyl methanesulfonate-mutagenized cultures (3, 4), their independent origin could not be ascertained. From each clone we selected two subclones resistant to medium containing 10 times more inhibitor (5 μg/ml; step II). A third selection with 25 μg of coformycin per ml yielded one step III mutant from each step II line. The lineages and relative levels of AMPD activity of these clones are reported in Fig. 1.

Sodium dodecyl sulfate-polyacrylamide gel analysis of the protein content of step III lines. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) showed the pattern of proteins in crude extracts from five step III mutants and the reference line GMA32. A band of relatively constant intensity migrating like purified AMPD was observed in extracts from the five mutant lines (molecular weight, 120,000). An intense band was present at the position of X (molecular weight, 60,000), and a faint band was present at the position of W (molecular weight, 35,000) in extracts of lines $HC_{50}422$ and $HC_{50}611$ only. At the position designated Y1,Y2,Y3 (molecular weight, around 25,000), a complicated

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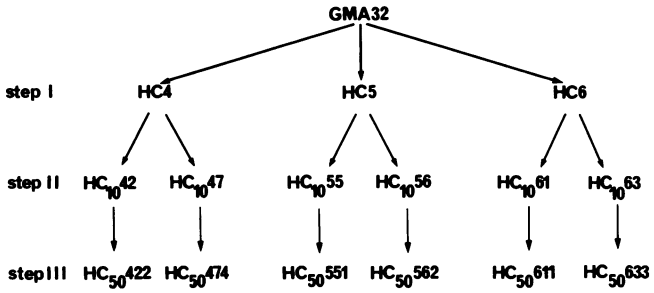

FIG. 1. Derivation of cell lines. The relative AMPD activities were 1 in the wild-type GMA32 line, 7 to 10 in step I, 30 to 40 in step II, and 100 to 150 in step III lines.

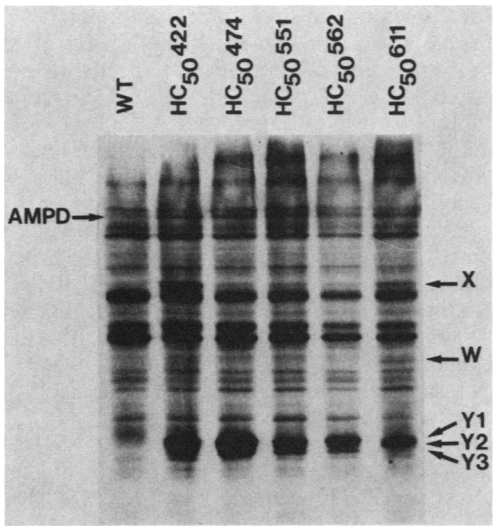

FIG. 2. Sodium dodecyl sulfate-polyacrylamide (12.5%) gel electrophoresis of crude extracts from wild-type (WT) and five highly resistant lines grown in the presence of [^{3}H]leucine. The arrows indicate the positions at which AMPD and other associated proteins (X, W, Y1, Y2, and Y3) were detected.

but reproducible pattern of proteins was observed: at least three bands could be recognized. The Y1 protein accumulated in all mutant lines but at different levels; the intensity of this band was remarkably high in $HC_{50}474$ and low in $HC_{50}611$. The Y2 protein also appeared to accumulate in all mutant lines (see below). The Y3 protein was abundant in the four lines derived from HC4 and HC5 but was undetectable in $HC_{50}611$.

Dot blot quantification of poly(A)$^+$ mRNAs encoding the X, W, Y1, and Y2 proteins in step III variants. cDNA probes are available for the genes encoding the X, W, Y1, and Y2 proteins in step III variants. As previously shown (5), probes H3, H4, and H6, constructed from poly(A)$^+$ mRNA of line $HC_{50}611$, are complementary to mRNAs coding for W, X, and Y2 proteins, respectively; probe H11, constructed from poly(A)$^+$ mRNA of line $HC_{50}474$, is complementary to Y1 mRNA. Results of dot blot hybridizations of the probes to known amounts of poly(A)$^+$ RNAs from the mutants and the wild-type line are shown in Table 1. Upon hybridization, the W probe yielded a signal 32 and 128 times stronger with $HC_{50}422$ and $HC_{50}611$ mRNAs, respectively, than with wild-type mRNAs. In contrast, it was only 5 to 6 times stronger with RNAs from $HC_{50}551$ and $HC_{50}562$, and $HC_{50}474$ yielded a wild-type signal. Similar results were obtained by using total instead of poly(A)$^+$ RNAs (data not shown). These results account for the observed amount of W protein in extracts of the corresponding lines. The accumulation of mRNA hybridizing to the X probe followed the same kind of distribution, in complete agreement with the amount of X protein accumulated in extracts of the mutants. The mRNA complementary to the Y1 probe was more abundant in line $HC_{50}474$, which also contained the highest amount of Y1 protein, and its accumulation in the other lines is consistent with accumulation of this protein. The pattern of Y2 protein in the different mutant extracts did not correlate entirely with the relative amount of mRNA detected by the Y2 probe. This probe did indeed detect mRNA accumulation in $HC_{50}611$ and $HC_{50}422$, which overproduce Y2 protein, but a protein band migrating like Y2 was visible in extracts of $HC_{50}551$, $HC_{50}562$, and $HC_{50}474$, despite the very low accumulation of Y2 mRNA in these lines. This situation is likely to result from the migration of two proteins at the same level on the gel. One is the product of the gene detected by Y2, while the other one is a minor protein detected after in vitro translation of the mRNAs hybridized to the Y1 probe (5). This interpretation is consistent with the fact that the same paradoxical observation was made in three lines accumulating large amounts of the Y1 protein and mRNA. Two-dimensional gel electrophoresis is in progress to clarify this point. With this apparent exception, the differences observed in the abundances of W, X, Y1, and Y2 proteins are the direct manifestation of fluctuations in specific mRNA contents.

Dot blot quantification of the number of genes encoding W, X, Y1, and Y2 proteins in step III mutants. To determine whether the accumulation of specific mRNAs homologous to probes W, X, Y2, and Y1 in these mutant lines is fully accounted for by gene amplification, we quantified the

TABLE 1. Relative levels of overproduced proteins (P), mRNAs, and DNAs in highly amplified mutants[a]

Cell line	AMPD	W:H3			X:H4			Y2:H6			Y1:H11		
	P	P	RNA	DNA	P	RNA	DNA	P	RNA	DNA	P	RNA	DNA
$HC_{50}422$	+	+	32	50	++	32	50	+	16	16	++	64	32
$HC_{50}474$	+	−	1	150	−	1	1	+	1	1	+++	128	64
$HC_{50}551$	+	−	6	50	−	4	5	+	4	4	++	64	32
$HC_{50}562$	+	−	6	50	−	4	5	+	4	4	++	32	32
$HC_{50}611$	+	++	128	200	++	64	50	+	64	64	+	16	8
GMA32	−	−	1	1	−	1	1	−	1	1	−	1	1

[a] Amounts of protein were estimated from the intensities of corresponding bands in sodium dodecyl sulfate-polyacrylamide gels. −, Not detectable; +, detectable; ++, abundant; +++, very abundant. Relative RNA and DNA levels were determined by spot blot experiments, taking the wild-type GMA32 content as 1.

number of copies of the four corresponding genes in each mutant (Table 1; Fig. 3). The number of copies of genes X, Y2, and Y1 was in good agreement with the abundance of the corresponding mRNAs in all lines tested. In contrast, a marked difference was observed when the level of amplification of the W gene was compared with the levels of the specific mRNA and the W protein in the three lines $HC_{50}474$, $HC_{50}551$, and $HC_{50}562$. Although this gene was amplified 50- to 150-fold, no accumulation of the corresponding mRNA was detected in $HC_{50}474$, and only a very moderate sixfold increase was found in $HC_{50}551$ and $HC_{50}562$. As shown below, alterations in the structure of this gene are responsible for defective transcription.

Restriction analysis of amplified sequences in step III mutants. DNA samples from the mutants and the wild-type line GMA32 were digested with restriction enzymes *Pst*I, *Eco*RI, *Sac*I, *Bam*HI, and *Pvu*II and analyzed by the Southern blotting procedure (9). Within the limits of sensitivity of this method, very few rearrangements were detected in genes X, Y1, and Y2 (for an example, see Fig. 3, panels A2 and B2).

The distribution of DNA fragments detected in gene W was more complex. With most restriction enzymes, fragments not detected upon digestion of the GMA32 DNA appeared (Fig. 3, panels A1 and B1) in the digests of the mutant DNAs. Some of these additional fragments (for example, fragment 7.5 Kb on Fig. 3, panel A1) were observed in clones from different lineages generated through several cycles of amplification. Their origin remains to be established; they may be present in the wild-type line but homologous to only a small part of the probe and thus not detected in the wild-type DNA.

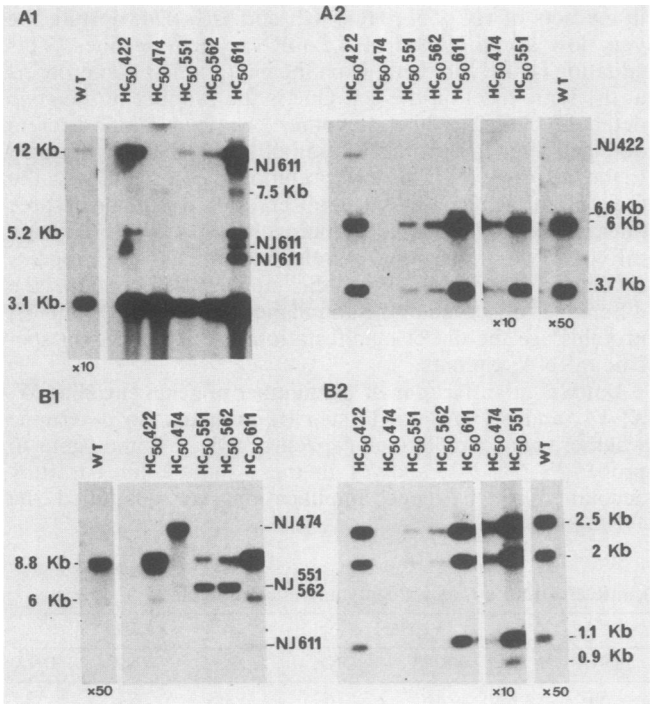

FIG. 3. Blot analysis of DNAs from step III mutants and the wild-type (WT) line. Each DNA (10 μg) was digested with *Eco*RI (panels A1 and A2) or *Pst*I (panels B1 and B2), fractionated in a 0.7% agarose gel, and transferred to nitrocellulose. The filters were hybridized with nick-translated W probe (panels A1 and B1), eluted, and rehybridized with Y2 probe (panels A2 and B2). The designations ×10 and ×50 indicate corresponding overexposures of autoradiograms. Molecular sizes are expressed in kilobases (kb). NJ, Novel joint.

TABLE 2. Amplification levels (fold) of the three wild-type *Eco*RI fragments in the highly amplified cell lines[a]

Cell line	3.1 kb	5.2 kb	12 kb
$HC_{50}422$	50–75	30–60	30–60
$HC_{50}474$	150	1–2	1–2
$HC_{50}551$	50	5	5
$HC_{50}562$	50	5	5–10
$HC_{50}611$	200	200	200

[a] Three different *Eco*RI blots of DNAs from the highly amplified lines (hybridized to the W gene probe) were exposed for various periods of time and scanned with a Vernon photometer. We combined these data with the results of dot blot analysis (Fig. 1) to calculate the amplification levels.

Some new fragments were specific for one line, for example, NJ611 and NJ474 (Fig. 3, panels A1 and B1). The specificity indicates that they are manifestations of gene rearrangements. The same explanation must also be true for the additional fragment NJ551,562 common to lines $HC_{50}551$ and $HC_{50}562$, which were derived from the same HC5 step I line (see below). Some of these novel joints (Fig. 3, panel B) appeared to be present in only one or a few copies (NJ611), whereas some were highly amplified (NJ474 and NJ551,562). The latter two rearrangements were characterized by the appearance of new fragments only upon *Pst*I digestion. With other enzymes, these novel joints were detected by an unequal degree of amplification of the wild-type fragments. Thus, upon *Eco*RI digestion (Fig. 3, panel A1), only the 3.1-kilobase (kb) wild-type fragment was amplified (×150) in $HC_{50}474$. In lines $HC_{50}551$ and $HC_{50}562$, this fragment was amplified about 50 times, whereas the 5.2- and 12-kb fragments were amplified 5- to 10-fold (Table 2). This showed that the 5.2- and 12-kb *Eco*RI fragments were absent from all ($HC_{50}474$) or most ($HC_{50}551$, $HC_{50}562$) amplified units, whereas the 3.1-kb fragment, which was always amplified at a high level, was retained in all these units. Therefore, a rearrangement must have separated the 3.1-kb fragment from the exons of the 5.2- and 12-kb *Eco*RI fragments, which were excluded from the amplified unit. It implies that the novel joints detected upon *Pst*I digestion are complementary to the 3.1-kb *Eco*RI fragment. This is indeed the case, because (i) in the three highly amplified lines, the amplification levels of the 3.1-kb fragment paralleled the amplification levels of the novel joints NJ474 and NJ551,562, which was not true for the 5.2- and 12-kb *Eco*RI fragments, and (ii) hybridization experiments (data not shown) confirmed that the same subfragment of the W probe was specific for both the 3.1-kb *Eco*RI fragment and the novel joints. Since we used a full-length cDNA probe, this situation is best explained if the two rearrangements generating NJ474 and NJ551,562 upon *Pst*I digestion took place in the same intron because, as explained in the Discussion, rearrangement within an exon would result in a detectable novel fragment, whichever restriction enzyme is used.

Restriction analysis of amplified sequences in mutants of the same lineage. To gain information on the origin of highly amplified novel joints, we analyzed the structure of the W gene in step I and II mutants from which $HC_{50}474$, $HC_{50}551$, and $HC_{50}562$ were derived. As an example, Fig. 4 shows the results obtained with HC5 and its derivatives. The multiple copies of the novel joint observed in $HC_{50}551$ and $HC_{50}562$ did not accumulate during a single amplification step; the rearrangement was generated at the first step. It was

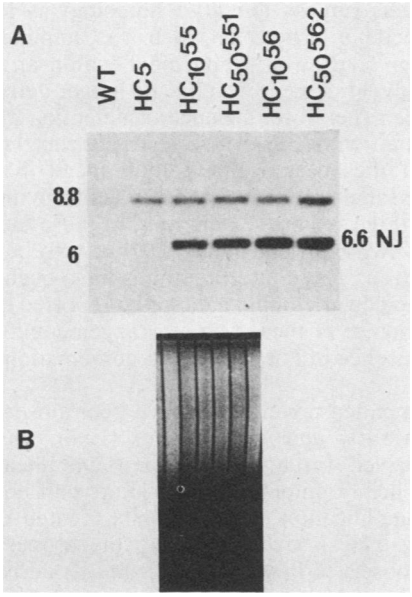

FIG. 4. Blot analysis of DNAs prepared from the wild type (WT) and HC5 and its derivatives. (A) Each DNA (10 μg) was digested with *Pst*I, treated as described in the legend to Fig. 3, and hybridized with nick-translated W probe. Molecular sizes are expressed in kilobases. (B) Ethidium bromide staining of the gel. NJ, Novel joint.

reamplified during the second step, whereas the wild-type W gene fragments were not. During the third step, neither the novel joint nor the wild-type W fragments were reamplified, suggesting that both the wild-type and rearranged copies of this gene were excluded from newly amplified units.

This analysis also revealed that some DNA sequences were lost after subsequent amplification steps. Thus, most wild-type copies of W gene detected in line $HC_{10}47$ disappeared in line $HC_{50}474$ (Fig. 5).

DISCUSSION

We have shown (5) that several proteins with unknown biological activities coaccumulate with the selected AMPD enzyme in a coformycin-resistant mutant ($HC_{50}611$), and we demonstrated that gene amplification was responsible for overproduction of four of these proteins, designated X, W, Y1, and Y2. Because no probe for AMPD is yet available, amplification of this gene has not been demonstrated directly. However, the genetic properties of the mutants selected for AMPD overproduction (3, 4) indicate that the same molecular mechanism is responsible for the accumulation of this enzyme as well as the X, W, Y1, and Y2 gene products.

In the present work, we compared the level of amplification and the structure of the coamplified genes in mutants of different lineages. Overproduction of unselected proteins through gene amplification can be a manifestation of genetic linkage, since it is a general observation (1, 6, 7, 11) that amplified DNA segments are far larger than the selected gene. Alternatively, it might be imposed by the necessity to compensate for the toxicity of the increased level of the selected enzyme (2). In the present work, we showed that the X, Y1, and Y2 genes were amplified in some but not all AMPD-overproducing lines. The W gene was amplified in all lines, but the amplified copies were expressed only in two of them. The observation that none of the four associated proteins accumulated in all mutants rules out the possibility that their overproduction is physiologically required for the survival of cells with a high level of AMPD activity.

The properties of the W gene raise a particular problem. As just pointed out, in contrast to the other genes studied, it was amplified in all mutants. Moreover, in all lines its amplification level was close to the level expected for the AMPD gene itself. This suggests its tight linkage with the selected gene. The mutants distribute into two categories according to the expression of the W gene. In the first ($HC_{50}611$ and $HC_{50}422$), all or most copies of W were expressed and the Y2 and X genes were amplified to high levels. In the second, no ($HC_{50}474$) or few ($HC_{50}551$ and $HC_{50}562$) copies of W were expressed, because transcription was turned off by a rearrangement within the gene. The Y2 and X genes were then unamplified or weakly amplified, in parallel with the number of functional copies of the W gene. Conceivably, accumulation of the W protein could be toxic and impose the selection for clones also overproducing the X and Y2 gene products. Alternatively, X, Y2, and W might be genetically linked in such a way that rearrangements in W exclude X and Y2 from amplified units. The latter hypothesis is supported by the results of in situ hybridization experiments (B. Robert de Saint Vincent, manuscript in preparation) indicating the same chromosomal location for amplified copies of W, X, and Y2 genes in line $HC_{50}611$.

Assuming that all the coamplified genes are linked to the AMPD gene, the observation that they were not amplified to the same level in the same line indicates that, as previously observed for CAD (1) and dihydrofolate reductase (6) gene amplification, the amplification process does not generate identical units. The segregation pattern of the amplified genes in the various lines (Table 1) further allows us to propose a genetic map of the amplifiable region. We have presented evidence above suggesting a close linkage of the W and AMPD genes. Gene Y1 was weakly amplified in line $HC_{50}611$, on which genes X and Y2 were amplified. In contrast, gene Y1 was amplified in the lines which do not amplify X and Y2. This segregation pattern is consistent only with the location of the AMPD and W genes between the X

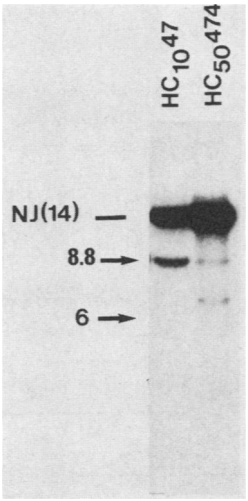

FIG. 5. Blot analysis of DNAs prepared from the step II line $HC_{10}47$ and its step III derivative $HC_{50}474$. Each DNA (10 μg) was digested with *Pst*I, treated as described in the legend to Fig. 3, and hybridized with nick-translated W probe. The equivalence of DNA amounts in the gel was checked by ethidium bromide staining. Molecular sizes are expressed in kilobases. NJ, Novel joint.

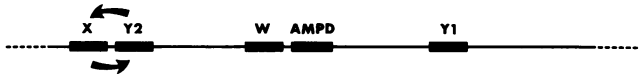

FIG. 6. Genetic map of the W, X, Y1, Y2, and AMPD genes suggested by their segregation pattern during amplification.

and Y2 group and the Y1 gene. Moreover, the observation that gene W was rearranged in the three lines which did not amplify the X and Y2 genes suggests that W is located between AMPD and the X and Y2 gene pair. In line $HC_{50}611$, in which the AMPD and W genes were amplified, the Y1 gene was weakly amplified, indicating that gene Y1 is not located between the AMPD and W genes. The resulting genetic map is represented in Fig. 6.

Our analysis revealed several novel joints in the DNAs of the amplified lines. They were found in four of five lines under investigation when the W gene was probed, whereas probes specific for X, Y1, and Y2 genes detected very few rearrangements. These three probes together screened the structure of about 50 kb of DNA versus about 35 kb for the W probe (estimated by adding the lengths of the restriction fragments detected in Southern blots). Thus, the distribution of novel joints along the amplified DNA appears to be nonrandom. Two interpretations can account for this observation. If, as previously considered, overproduction of the W gene product is toxic, rearrangements in W gene would be selected for. Alternatively, clustering of novel joints may be directly related to the molecular mechanisms generating the amplified sequences or to the presence of hot spots for their recombination. Genomic libraries are presently used to determine the precise location of the two highly amplified novel joints, NJ474 and NJ551,562, identified in gene W. The data presented above suggest that these rearrangements take place in the same intron. The W probe we used is a full-length cDNA with no *Eco*RI restriction site; therefore, each *Eco*RI fragment it detects contains one or several complete exons. Any rearrangement occurring within an exon would split the corresponding *Eco*RI fragment to generate a novel *Eco*RI fragment which still bears part of this exon and thus (unless too little homology is left) should remain detectable, especially when it is amplified (Fig. 7). On the other hand, a rearrangement within an intron can result in a novel fragment which is no longer detectable by a cDNA probe. Therefore, absence of detection of an amplified novel joint with four of five restriction enzymes is best explained if the rearrangement took place in an intron. Because the same fragments are excluded from the amplified units of the three cell lines, whichever of these four enzymes is used, the two rearrangements are likely to be located in the same intron. These interpretations have been confirmed by recent molecular cloning data (to be reported elsewhere). The close linkage of these two rearrangements further suggests the presence of hot spots for recombination within the W gene.

Highly amplified novel joints have been observed in this and in previously analyzed systems (1, 6). The study of mutants derived through successive amplification steps shows that highly amplified novel joints can be generated through reamplification of novel joints created earlier as a single copy. This is exemplified by the properties of the novel joint observed in mutant HC5 and its derivatives.

During this study, we also observed that the amplification process is more complex than the mere addition of newly generated units to preexisting DNA. Some of the wild-type copies of gene W (about 10) present in line $HC_{10}47$ were no longer detected in its $HC_{50}474$ derivative; instead, rearranged copies of this gene accumulated (Fig. 5). We did not detect double minute structures at the various stages of amplification of AMPD-overproducing lines; on the contrary, as pointed out above, amplified copies of gene W have been shown to be chromosomal. This indicates that changes in the distribution of gene W copies are not accounted for by selection of double minute subpopulations. The block substitution of modified amplification units to preexisting units has been reported in cells with amplified transfected DNA. Roberts and Axel (8) observed that a tandem of amplified units was replaced by the same number of units altered by the same deletion or point mutation. Subramani and Rubnitz (10) have also shown that several amplification units ap-

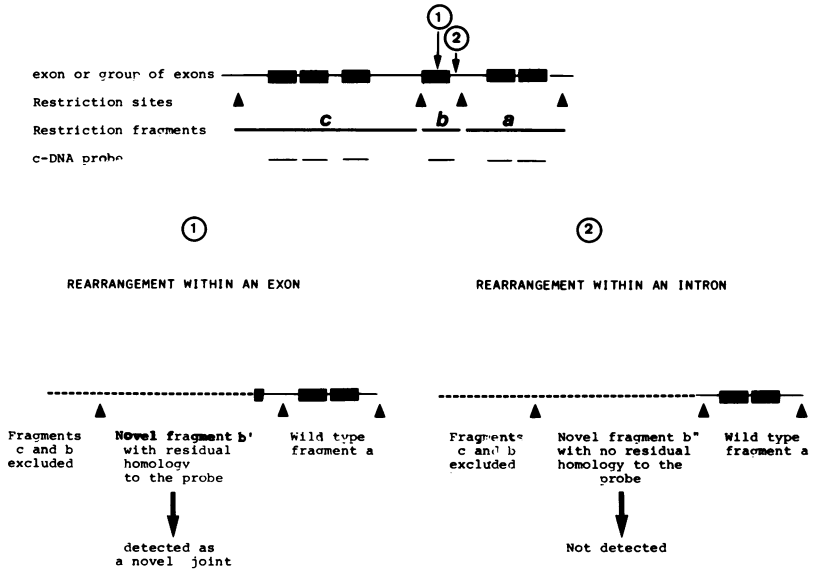

FIG. 7. Consequences of the location of a rearrangement on the detection of a novel fragment with a cDNA probe. ----, Recombined sequence of unknown origin.

peared to have deleted a marker gene in an identical fashion. Gene conversion was considered as a plausible mechanism to account for these observations. The present work indicates that apparently similar phenomena can be observed during amplification of endogenous DNA. Gene conversion might also explain these results. Alternatively, double homologous recombinations could account for our results. At least in some cases, reamplification appears to involve a single preexisting unit. As an example, in the selection of mutants with a higher level of resistance from line HC5 (Fig. 4), only the unit identified by the presence of the joint was reamplified. If the unit selected for reamplification is at the origin of tandem repeats of transiently extrachromosomal copies, reintegration of these tandems may occur through double recombinations which will more or less completely substitute reamplified copies for preexisting ones. This can account for the deletion of wild-type copies observed in line $HC_{50}474$ (Fig. 5).

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