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Expression of several amplified genes in an adenylate-deaminase overproducing variant of Chinese hamster fibroblasts

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Unstable variants with increasing amounts of adenylate-deaminase (AMPD) have been stepwise recovered from Chinese hamster fibroblasts plated in selective medium containing increasing coformycin concentrations; several polypeptides accumulate in the variants in parallel to AMPD: they are no longer detectable in cells which reverted to the wild-type enzyme level. We report here the molecular cloning of cDNA sequences complementary to mRNAs coding for four such polypeptides. The plasmidic probes have been exploited to characterize their complementary mRNAs and to quantify the copies of these cognate genes in a variant and in two revertant clones. The results show that different mRNAs code for the four polypeptides; their accumulation is accounted for by amplification of their specific genes; these observations suggest that cells overproducing AMPD are characterized by the presence of amplification units comprising several expressed genes.

Key words: adenylate-deaminase/Chinese hamster fibroblasts variants/coformycin/gene amplification

Introduction

We have shown that unstable variants with increased adenylate-deaminase (AMPD) activity can be isolated from the GMA32 Chinese hamster fibroblastic line growing in medium supplemented with coformycin, adenine and azaserine (Debatisse *et al.*, 1982). Stepwise selection in the presence of increasing coformycin concentrations yielded variants with progressively higher activity of the enzyme. The correlation of enzyme activity with the intensity of a protein band co-migrating with purified AMPD during SDS-polyacrylamide gel electrophoresis of cell extracts established that AMPD hyperactivity is the manifestation of overaccumulation of the enzyme protein. The similarity between these properties and those of drug-resistant lines shown to have an increased number of copies of the gene coding for the drug target enzyme (Alt *et al.*, 1978; Wahl *et al.*, 1979; Beach and Palmiter, 1981; Yeung *et al.*, 1983; Sanders and Wilson, 1984) strongly suggested that the coformycin-resistant variants also resulted from gene amplification. A remarkable property of this system is that several proteins other than AMPD jointly accumulate through the successive steps of selection and return to wild-type level in revertant clones (Debatisse *et al.*, 1982).

Here we analyze the molecular mechanisms leading to accumulation of AMPD and associated proteins in the resistant cells. Several cDNAs complementary to mRNA coding for overproduced proteins have been cloned and used to demonstrate that accumulation of these proteins – and presumably of AMPD itself – is due to gene amplification.

Results

Polypeptides accumulated in the AMPD overproducing line $HC_{50}611$

A line, $HC_{50}611$, which expresses 150-fold more AMPD activity than wild-type GMA32 cells, was isolated through three steps of selection in medium containing azaserine, adenine and increasing coformycin concentrations (Debatisse *et al.*, 1982). SDS gel electrophoresis of proteins present in this variant shows one discrete band which has a pattern of migration identical to that of purified AMPD. This band is not present in GMA32 extracts (Debatisse *et al.*, 1982 and Figure 1), demonstrating that increased AMPD activity is the consequence of an overaccumulation of enzyme molecules. Other bands of markedly increased intensity are also observed at the levels designated as X, W and nY in extracts of $HC_{50}611$ (the symbol nY is used to underscore the fact that several bands can be recognized at that level; see below). None of these polypeptides appears to be structurally related to AMPD: immunodetection of AMPD antigenic determinants by a rabbit antiserum raised against the purified enzyme (not shown) clearly reveals the AMPD band on a Western blot of denaturing gels of the $HC_{50}611$ extract, but no other overproduced

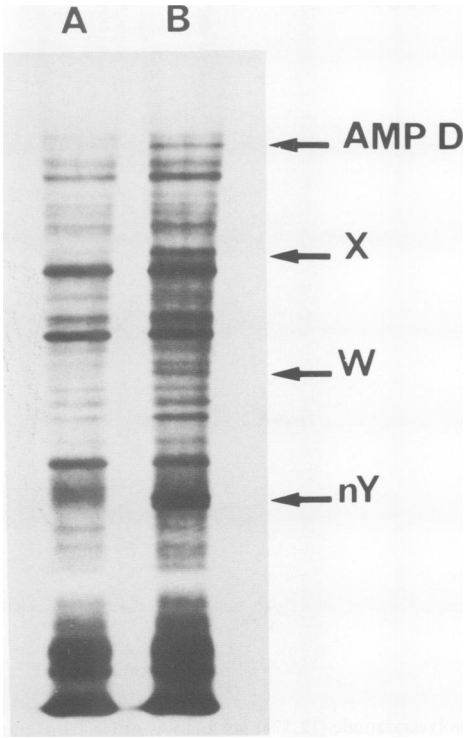

Fig. 1. SDS-polyacrylamide (12.5%) gel analysis of cell extracts: **(A)** = GMA32 (wild-type); **(B)** = $HC_{50}611$. Arrows locate proteins overaccumulated in the mutant extract.

polypeptide, despite their greater abundance.

In vitro translation products of poly(A)$^+$ mRNAs from line $HC_{50}611$

We compared the *in vitro* translation products of poly(A)$^+$ mRNAs prepared from the GMA32 and from the $HC_{50}611$ line. We observed (Figure 2) a markedly enhanced synthesis of the X, W and nY polypeptides but no evidence for AMPD synthesis in these experiments. Repeated attempts to immunoprecipitate *in vitro* translation products with the antiserum raised against purified AMPD were unsuccessful. We tried several conditions for AMPD translation, including translation of total rather than poly(A)$^+$ RNA and changes in the $KCl/MgCl_2$ ratio with negative results. The use of *Xenopus* oocytes as a translation system did not allow the detection of increased protein synthesis in the variant at the level expected for AMPD. Thus, whether AMPD mRNA is present in these preparations is uncertain (see Discussion), but in contrast mRNAs encoding X, W and nY proteins are present among $HC_{50}611$ poly(A)$^+$ RNAs and their translation yields increased amounts of these polypeptides.

Identification of the products of the mRNAs selected by plasmidic probes

Four plasmids – designated H3, H4, H6 and H11 – carrying inserts of cDNA from $HC_{50}611$ which did not cross-hybridize and hybridized more strongly with $HC_{50}611$ DNA

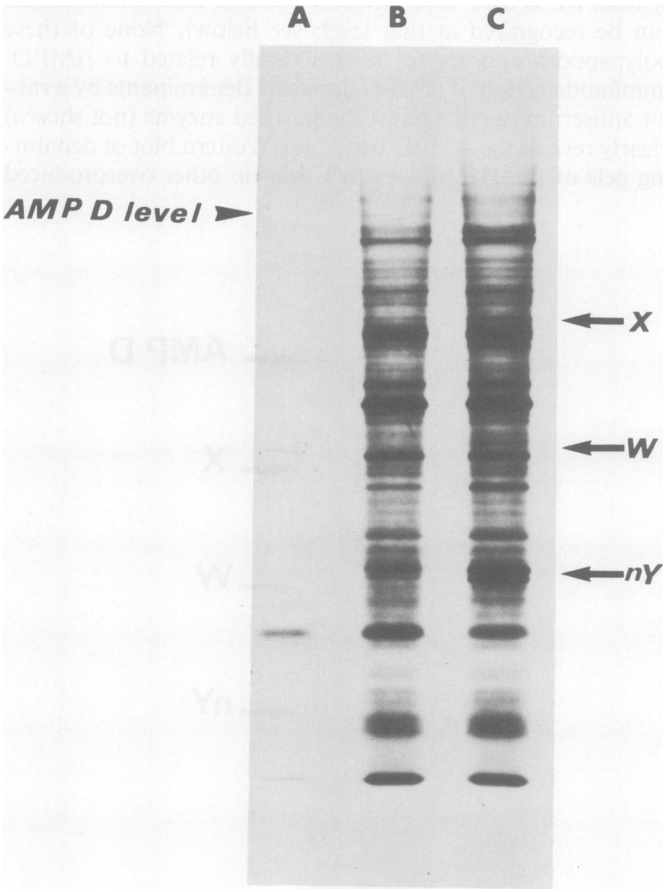

Fig. 2. SDS-polyacrylamide (12.5%) gel analysis of '*in vitro*' translation products. **(A)**: no exogenous RNA added; **(B)**: GMA32 poly(A)$^+$ mRNA added; **(C)**: $HC_{50}611$ poly(A)$^+$ mRNA added.

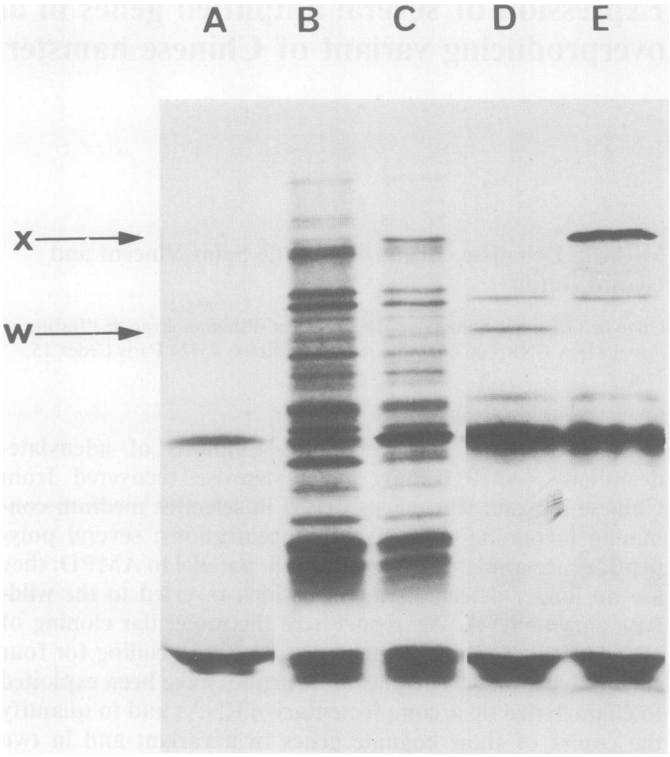

Fig. 3. Translation products of mRNAs selected by plasmids H3 and H4. **Lane A:** lysate, no exogenous RNA added; **lane B:** total poly(A)$^+$ RNA from GMA32 added; **lane C:** total poly(A)$^+$ RNA from $HC_{50}611$ added; **lane D:** RNA selected by H3 added; **lane E:** RNA selected by H4 added.

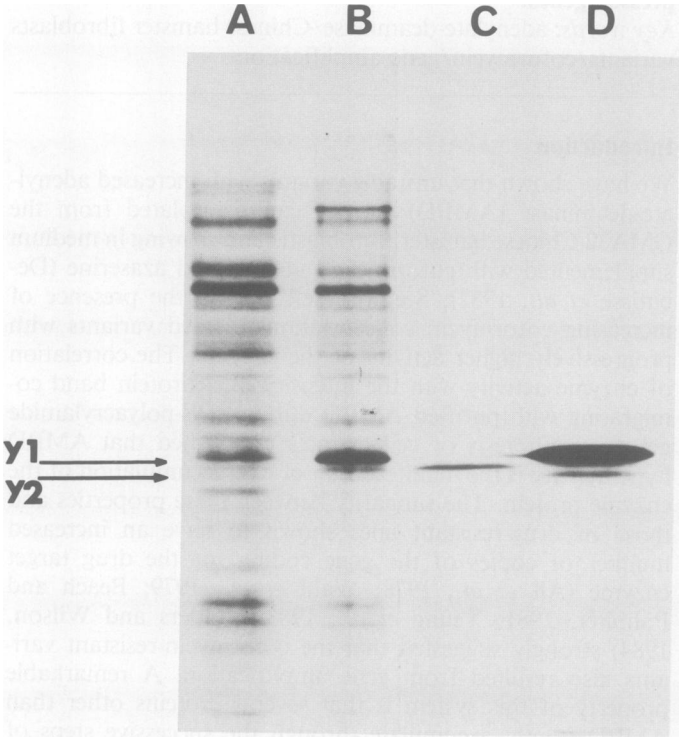

Fig. 4. '*In vitro*' translation products of mRNAs selected by plasmids H6 and H11. **Lanes A** and **B:** translation products of total poly(A)$^+$ mRNA from GMA32 and $HC_{50}611$, respectively. **Lanes C** and **D:** translation products of mRNA eluted from plasmids H6 and H11, respectively.

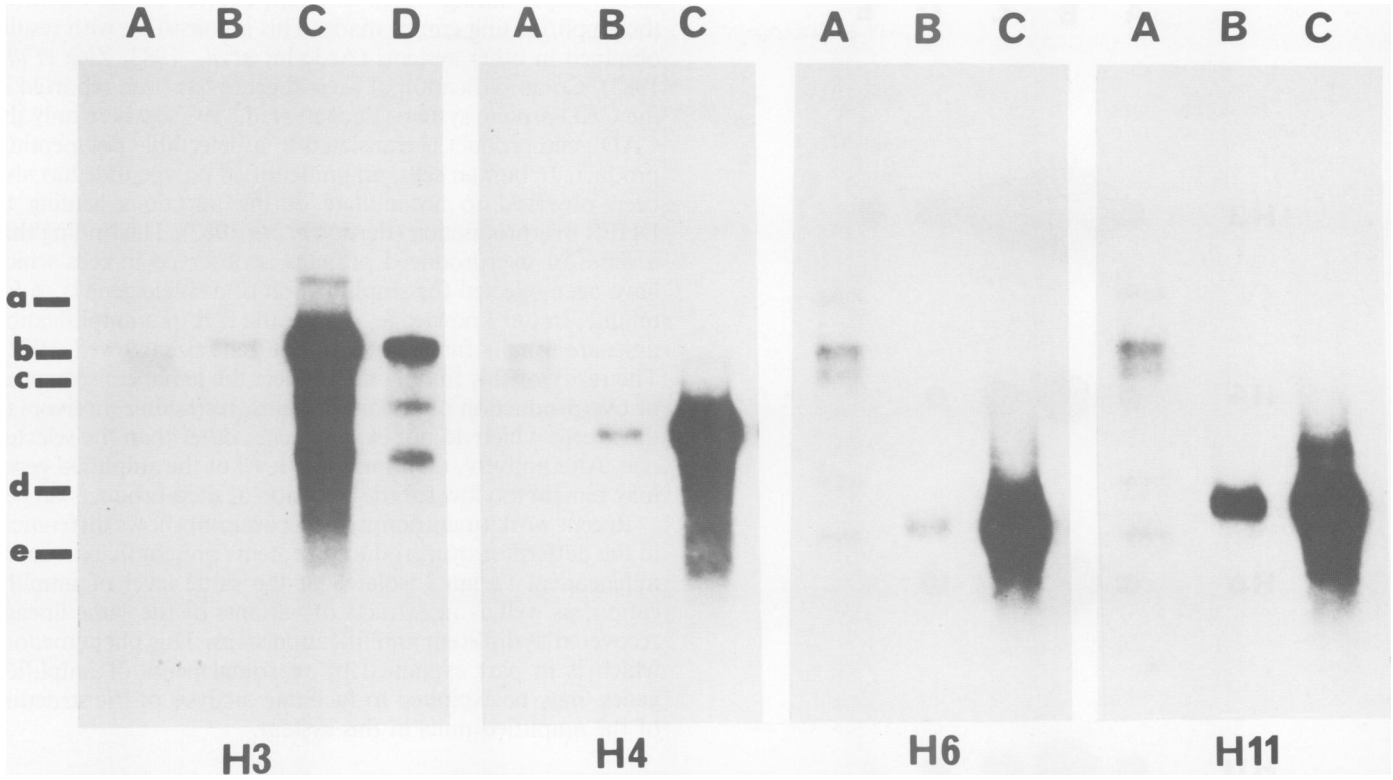

Fig. 5. Northern blot analysis of GMA32 and $HC_{50}611$ mRNA. Poly(A)$^+$ RNAs were denatured with glyoxal, separated by agarose gel electrophoresis and transferred on 'gene screen' membrane (NEN). Filters were hybridized with nick-translated probes H3, H4, H6 and H11. **Lane A:** mol. wt. markers (**a:** 4.362 kb; **b:** 3.256 kb; **c:** 2.819 kb; **d:** 1.543 kb; **e:** 1.106 kb). **Lane B:** 2 μg of GMA32 poly(A)$^+$ RNA; **lane C:** 2 μg of $HC_{50}611$ poly(A)$^+$ RNA; **lane D:** 0.4 μg of $HC_{50}611$ poly(A)$^+$ RNA.

than with GMA32 DNA were constructed (see Materials and methods). DNA from these plasmids was bound to nitrocellulose and the filters were used to select complementary RNAs (Parness *et al.*, 1981) from total poly(A)$^+$ RNAs of the $HC_{50}611$ line. The RNAs complementary to the plasmids were eluted and translated *in vitro*; the translation products were analyzed on an SDS-polyacrylamide gel (Figures 3 and 4). These experiments show that the H3 insert is complementary to an RNA coding for a product which migrates similarly to the W polypeptide; the H4 insert is likewise related to a protein which migrates similarly to that of the X polypeptide; H6 to a protein of the Y group (designated Y2); H11 to the major protein of the Y group (Y1) and to two proteins of lower apparent mol. wt. Whether the latter proteins are degradation products of Y1 or translation products of mRNAs selected with Y1 mRNA by this probe remains to be determined.

Expression in the wild-type and $HC_{50}611$ lines of mRNAs homologous to the cDNA probes

We compared by Northern analysis the abundance of mRNA hybridizing to the different probes in poly(A)$^+$ mRNA made from wild-type and variant cells. As shown in Figure 5, all plasmids recognize RNA species in greater quantity in $HC_{50}611$ than in GMA32. The RNA hydridization pattern is specific for each probe, confirming that the various probes carry DNA sequences belonging to different genes. The hybridization pattern of H3 is complex, with a major band at 3500 kb; H4, H6 and H11 hybridize to 2.0-kb, 1.2-kb and 1.3-kb RNAs, respectively.

Abundance in the wild-type and $HC_{50}611$ genomes of the sequences homologous to the cDNA probes

Dot blots of genomic DNA from GMA32 and $HC_{50}611$ were hybridized to nick-translated DNA from the various probes.

H3, H4, H6 and H11 probes all yield much stronger signals with $HC_{50}611$ genomic DNA than with equivalent amounts of genomic DNA from GMA32 (Figure 6). We can roughly estimate the amplification factor for each gene: H3 carries a sequence amplified ~125 times, H4 at least 25 times, H6 ~75 times and H11 ~15 times in the variant. These amplification factors are in close agreement with the increased levels of RNAs observed in the variant line in the Northern blot experiments. We also examined the number of gene copies homologous to H3, H4 and H6 probes in the genomic DNA of two revertant clones – designated 61-11 and 61-8 – isolated upon extensive growth in non-selective medium (Debatisse *et al.*, 1982): in both clones, which recovered coformycin sensitivity and wild-type level of AMPD activity, the estimated number of copies of the genes corresponding to the three probes was the same as in the wild-type line (data not shown).

Discussion

Analysis by SDS gel electrophoresis of the proteins present in extracts of the coformycin-resistant line $HC_{50}611$ shows that a set of at least four polypeptides co-accumulate with the AMPD protein. These polypeptides – W, X, Y1 and Y2 – are also overproduced upon *in vitro* translation of $HC_{50}611$ poly(A)$^+$ RNA. This observation suggests that accumulation

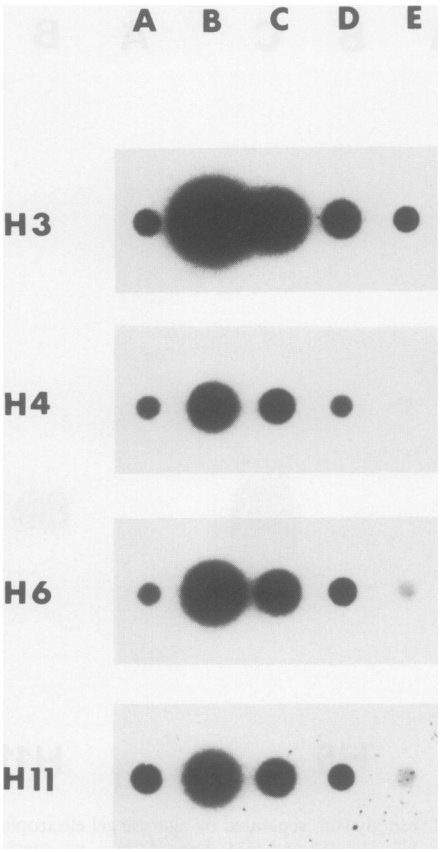

Fig. 6. Dot blot quantification of the relative copy number of DNA sequences hybridizing to H3, H4, H6 and H11 probes in GMA32 and $HC_{50}611$ lines (dot blots were made according to Kafatos *et al.*, 1979). **A**: 10 μg of GMA32 DNA; other dots correspond to $HC_{50}611$ DNA (**B** = 10 μg, **C** = 2 μg, **D** = 0.4 μg, **E** = 0.08 μg).

of the four proteins results from the greater abundance of their specific mRNA in the resistant line. This was indeed demonstrated by Northern blot analysis. We were unable, however, to characterize AMPD among the products of *in vitro* translation experiments; at least two interpretations can account for this particular result. It is possible that this mRNA may be poorly translated under our experimental conditions or, alternatively, it may be particularly unstable and lost during extraction. Work is in progress to clarify this issue.

The availability of plasmidic probes carrying genetic determinants specific for each of the four X, W, Y1 and Y2 polypeptides allowed us to show by dot blot experiments that the complementary DNA sequences are amplified. The four proteins are independent gene products: this conclusion can be drawn from the observation that the DNAs encoding the different polypeptides do not cross-hybridize but bind different RNA species present in extracts of the variant and – to a smaller extent – in extracts of the wild-type line; moreover, the level of amplification of the DNA sequences homologous to the various probes is significantly different. The most likely interpretation of these results is that coformycin resistance in variant $HC_{50}611$ is the manifestation of amplification of the AMPD gene, while overproduction of the various polypeptides results from co-amplification of genes associated within the same amplification unit. The observation that genes encoding W, X, Y1 and Y2 polypeptides are amplified to different levels may be explained by the finding that the resistance of the $HC_{50}611$ line was reached through several amplification steps, during which different choices of the extent of the amplified unit can be made. This is consistent with results obtained in other systems (Ardeshir *et al.*, 1983; Zieg *et al.*, 1983). Co-amplification of several genes has been reported in the CAD protein system (Padgett *et al.*, 1982), where only the CAD gene product is translated to a detectable polypeptidic product. In human cells, an unidentified polypeptide has also been observed to accumulate during selections leading to DHFR overproduction (Bertino *et al.*, 1982). The finding that a series of overproduced proteins is observed in cells which have been selected for amplification of a single gene is so far unique, to our knowledge, despite the fact that amplification units are usually far larger than one gene size (Cowell, 1982). The rarity of this finding may reflect the lethal consequences of overproduction of certain proteins, restricting survivors to those cells which do not express genes other than the selected one. Alternatively, the translation level of the amplified genes may remain too low for the detection of their products on gel.

Recent work (manuscript in preparation) shows differences in the pattern of overproduced proteins present in extracts of independent variants isolated at the same level of amplification, as well as in extracts of variants of the same lineage recovered at different amplification steps. This phenomenon, which is in part explained by rearrangements of amplified genes, may be exploited to facilitate analysis of the structure of the amplified units in this system.

Materials and methods

Cell lines

The GMA32 line of Chinese hamster fibroblasts and its coformycin-resistant $HC_{50}611$ derivative (initially designated as $HC_{50}61$) have been described previously (Debatisse *et al.*, 1982). $HC_{50}474$ is another coformycin-resistant clone isolated by the same protocol as $HC_{50}611$.

Isolation of poly(A)$^+$ RNAs

Cells were grown attached to plastic roller bottles (Nunc) in ERH medium supplemented with 10% foetal calf serum, antibiotics and the indicated supplements (Debatisse and Buttin, 1977). Medium was removed and the cells were quickly washed with phosphate buffered saline (PBS), trypsinized and pelleted (whole treatment: ~30 min). The pellet was resuspended (1 g of cells/20 ml) in urea (6 M), LiCL (3 M) and SDS (0.2%) and homogenized in a Waring blender at full speed for 1 min. The homogenate was kept overnight at 0°C, after which time the pellet was collected by centrifugation. All subsequent steps were performed according to Auffray and Rougeon (1980).

In vitro translation

The mRNA-dependent rabbit reticulocyte lysate was prepared and used for translations as described by Pelham and Jackson (1976), with the modifications introduced by Auffray and Rougeon (1980).

cDNA cloning

Avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences Inc.) was used to synthesize cDNA copies (Kafatos *et al.*, 1979) of total poly(A)$^+$ RNA from lines $HC_{50}611$ and $HC_{50}474$. After alkaline degradation of RNA templates, the complementary cDNA strands were synthesized with *Escherichia coli* DNA polymerase I (Klenow fragment, Boehringer) (Wickens *et al.*, 1978) and the resulting double-stranded cDNAs were treated with S1 nuclease (a generous gift of R.Nageotte) to remove single-stranded ends and to cleave the strand joining loops (Efstratiadis *et al.*, 1976). The cDNAs were fractionated on a sucrose gradient (Rougeon and Mach, 1977) and poly(dC) tails were added with terminal deoxynucleotidyltransferase (BRL) to those >600 bp. The cDNAs were annealed to plasmid pBR322 DNA, previously cleaved with endonuclease *Pst*I and extended with poly(dG) tails (NEN). The tetracycline ampicillin-sensitive *E. coli* strain 1838 (obtained from F.Rougeon) was used as a recipient for transfection. Two methods were exploited for the screening of recipients with plasmids carrying DNA sequences with different expression or dosage in wild-type *versus* resistant lines. In the first protocol (used to screen the $HC_{50}611$ library) poly(A)$^+$ RNAs from the wild-type or $HC_{50}611$ lines were used as templates for preparing ^{32}P-labelled (10^8 c.p.m./μg) cDNAs with reverse transcriptase; twin replica filters with cultures of plasmid-carrying cloned bacteria were hybridized (Grünstein and Hogness, 1975) to each cDNA probe (10^5 c.p.m./filter). Out of 600 clones examined, seven were found to bind more $HC_{50}611$ cDNA than cDNA from wild-type cells. These seven

plasmids distributed in three independent groups, according to their cross-hybridization patterns. One plasmid from each group was selected for subsequent work; these plasmids – designated below as H3, H4 and H6 – contain inserts of 3.4 kb, 0.6 kb and 0.8 kb, respectively. In the second protocol (used to screen the $HC_{50}474$ library), genomic DNAs were prepared from wild-type (GMA32) or variant ($HC_{50}474$) cells and spotted on nitrocellulose (2 μg/spot). Plasmidic DNAs were prepared by the alkaline lysis procedure from pools of four individual cultures of recombinant cDNA clones; these cDNAs were nick-translated and hybridized to filters spotted with genomic DNAs. Approximately 5% of the plasmid pools hybridized more strongly with DNA from $HC_{50}474$ than with DNA from GMA32. Hybridization with individual plasmids permitted the identification of those clones responsible for the differential signal. Most plasmids obtained by this protocol contained cross-hybridizing inserts. One plasmid, H11, containing a 1-kb insert, does not cross-hybridize with H3, H4 and H6 inserts.

Large-scale isolation of plasmidic DNA from the selected probes was performed according to the method of Godson and Vapnek (1973). Genomic DNA was prepared according to the method of Blin and Stafford (1976).

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