Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 337–341, January 1986
Biochemistry

Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells

(gene amplification/in-gel hybridization technique/cosmid cloning/adriamycin resistance/colchicine resistance)

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Communicated by S. E. Luria, September 23, 1985

ABSTRACT **The mechanism by which mammalian cells acquire resistance to chemotherapeutic agents has been investigated by using molecular genetic techniques. LZ and C5, two independently derived multidrug-resistant Chinese hamster cell lines, share specific amplified DNA sequences. We demonstrate that commonly amplified DNA sequences reside in a contiguous domain of ≈120 kilobases (kb). We report the isolation of this DNA domain in cosmid clones and show that the level of amplification of the domain is correlated with the level of resistance in multidrug-resistant cell lines. The organization of the amplified domain was deduced by a unique approach utilizing in-gel hybridization of cloned DNA with amplified genomic DNA. We show that the entire cloned region is amplified in adriamycin-resistant LZ cells and independently derived, colchicine-resistant C5 cells. A mRNA species of ≈5 kb is encoded by a gene located within the boundaries of this region. Genomic sequences homologous to the 5-kb mRNA span over 75 kb of the amplified DNA segment. The level of expression of this mRNA in multidrug-resistant cells is correlated with the degree of gene amplification and the degree of drug resistance. Our results strongly suggest that the 5-kb mRNA species plays a role in the mechanism of multidrug resistance common to the LZ and C5 cell lines.**

The use of cytotoxic agents in cancer therapy is often complicated by the emergence of multidrug-resistant tumor cells. Cultured mammalian cells that exhibit analogous multidrug-resistance phenotypes present an attractive model to study this phenomenon (1). It has been suggested that multidrug resistance is due to a decreased intracellular drug accumulation (2, 3) caused by alterations of the plasma membrane (4–6). Multidrug-resistant cells often contain double minute chromosomes or homogeneously staining chromosomal regions, suggesting that gene amplification at least in part underlies multidrug resistance (7–11).

Two independently derived multidrug-resistant Chinese hamster cell lines, C5 (4) and LZ (11), were used in our studies. LZ, a highly resistant line, and 77A, a weakly resistant line, were derived by stepwise selection with increasing concentrations of adriamycin from drug-sensitive V79 cells (11). C5, a highly colchicine-resistant line, was derived from drug-sensitive Chinese hamster ovary cells (12). C5 cells are cross-resistant to a number of cytotoxic agents, including adriamycin and colchicine. In our previous study, we used the technique of in-gel renaturation (13) to directly demonstrate the presence of amplified DNA sequences in the LZ and C5 genomes (14). It was also shown that a subset of amplified LZ DNA sequences was amplified in common between the two cell lines. One of the commonly amplified DNA fragments was cloned, and the degree of its amplification in the cells was shown to correlate with the degree of their drug resistance (14).

In the present study, using phage and cosmid vectors, we have cloned a contiguous domain of DNA sequences that spans ≈120 kilobases (kb) of the hamster genome and that is amplified in common in C5 and LZ cells. We have also developed a method based on in-gel DNA renaturation that allows the direct assignment of cloned DNA fragments to their amplified homologs in genomic DNA samples. We have identified, within the cloned domain, a gene that encodes a mRNA transcript of ≈5 kb, whose level of expression correlates with the degree of drug resistance of V79, 77A, LZ, and C5 cells. The gene encoding this mRNA is shown to span at least 75 kb of DNA within the amplified domain. Our data also suggest that this gene may be a member of a multigene family.

MATERIALS AND METHODS

Cell Culture. Chinese hamster cells were grown in minimal essential medium (GIBCO) lacking nucleosides and supplemented with 10% fetal calf serum and L-glutamine (2 mM). The medium for lines AuxB1 and its drug-resistant derivative CH^RC5 (a gift from V. Ling, University of Toronto) was supplemented with hypoxanthine (0.2 mM) and thymidine (0.04 mM). The medium for colchicine-resistant C5 cells contained colchicine (Sigma) at 5 μg/ml. The V79 cell line and its adriamycin-resistant derivatives 77A (low level of resistance), LZ (high level of resistance), and the revertant LZR (low level of resistance) were provided by N. Howell (University of Texas). The medium for 77A and LZ cells contained adriamycin (Adria Laboratories, Columbus, OH) at 0.1 μg/ml and 10 μg/ml, respectively.

Construction and Screening of Genomic Libraries. Genomic high molecular weight DNA (15) from LZ cells (100 μg) was partially digested with *Sau*3A and cloned into the *Bam*HI site of λ EMBL3 vector (16). The library was plated on *Escherichia coli* strain LE392 and duplicate sets of nitrocellulose filters were screened by plaque-hybridization (17). For cloning in cosmid vectors very high molecular weight DNA (100- to 200-kb fragment size) (18) was partially digested with *Mbo* I and cloned into the *Bam*HI site of cosmid vector pSAE (19). Cells of *E. coli* strain 1046 were transformed to ampicillin resistance, and duplicate sets of filters were screened by colony hybridization (20).

In-Gel Renaturation Analysis. Driver and tracer DNAs were digested with a 5-fold excess of *Bam*HI to assure complete digestion. Digested and purified tracer DNA was labeled with ^{32}P to ≈2×10^7 cpm/μg with T4 DNA polymerase and [α-^{32}P]dCTP (≈3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) (13). In control samples, 15 μg of LZ or V79 driver DNA was mixed with 3×10^6 (Fig. 2*a*) or 8×10^6 (Fig. 2*b*) cpm of LZ or V79 tracer DNA, respectively. In

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Abbreviation: kb, kilobase(s).

other samples, 10 ng of cosmid driver DNA was mixed with 3×10^6 cpm (0.2 μg) of either V79 or LZ tracer DNA and with 10 μg of *Eco*RI-digested total human DNA (a carrier).

The samples were electrophoresed at 50 V for 36 hr in a horizontal (30 × 24 × 0.4 cm) gel of 1% agarose in 22.5 mM Tris acetate, pH 8.3/10 mM sodium acetate/1 mM Na_2EDTA. After electrophoresis the gel was subjected to two cycles of in-gel renaturation and S1 nuclease digestion as described (16), with the following modifications: hybridization buffer was 50% formamide/5× concentrated SSPE (SSPE: 10 mM sodium phosphate, pH 7.0/0.18 M NaCl/1 mM Na_2EDTA) in Fig. 2*a* and 50% formamide/2× concentrated SSPE in Fig. 2*b*. The buffer volume used for washes was 400 ml per gel, and S1 nuclease digestion was done in 250 ml of digestion buffer containing 40,000 units of S1 nuclease (Sigma) after five 15-min washes with the digestion buffer. Following S1 nuclease treatment, the digestion products were eluted from the gel by washing with 300 ml of SSPE, pH 7.0/0.1% $NaDodSO_4$ for a total of 4 hr with six changes of buffer. Gels were dried and exposed with Kodak XAR-5 films at −80°C without screen.

Southern Hybridization. DNA, digested with *Bam*HI and electrophoresed on 0.8% or 1% agarose gels, was transferred (21) to a hybridization membrane (Zetabind, Bio-Rad) and hybridized for 16 hr in 0.75 M NaCl/75 mM sodium citrate, 5× concentrated Denhardt's solution (Denhardt's solution: 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% $NaDodSO_4$, and 10 μg of denatured salmon sperm DNA per ml to 1–3 × 10^6 cpm (<10 ng) of the indicated hybridization probe prepared as described (22). Blots were washed to a final stringency of 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 65°C and autoradiographed for 7 hr.

RNA Transfer Blot Hybridization. Total cellular or poly(A)$^+$ RNA prepared (23) was fractionated on denaturing (formaldehyde-containing) agarose gels and transferred to a hybridization membrane (GeneScreen*Plus*, New England Nuclear) by electroblotting in 25 mM sodium phosphate buffer (pH 7.0). DNA·RNA hybridization was carried out for 18 hr at 62°C in the presence of 1 M NaCl, 1% $NaDodSO_4$, 10% dextran sulfate, 10 μg of denatured salmon sperm DNA, and <10 ng of a ^{32}P-labeled hybridization probe (5×10^6 cpm) (22). Blots were washed to a final stringency of 30 mM NaCl/3 mM sodium citrate/1% $NaDodSO_4$ at 62°C followed by autoradiography.

RESULTS

Genomic Cloning and Chromosome Walking Experiments. DNA isolated from LZ cells was used to construct a library in λ EMBL3 vector. Thirty thousand recombinant phages were screened with plasmid pDR1.1 (13) as a hybridization probe. Six clones hybridizing to pDR1.1 were identified and proved to represent overlapping DNA fragments, each of which included a 1.1-kb *Bam*HI segment homologous to pDR1.1. The restriction map of two clones spanning 35 kb of DNA is presented in Fig. 1 (λ DRG2 and λ DRZ3). A 7.8-kb *Eco*RI fragment was subcloned into pUC18 (24). This single-copy DNA sequence was used to probe a cosmid library constructed from LZ DNA in cosmid vector pSAE. Eighteen positive clones were identified, their restriction maps were established, and fragments free of repeated sequences were again isolated and subcloned to yield plasmids pDR9.7 and pDR2.9. Four successive rounds of screening using "left side" probes pDR2.9 and pDR2.7 and "right side" probes pDR9.7, pDR1.6, and pDR2 (see Fig. 1) were performed and a total of 75 cosmids was isolated. The restriction map of DNA inserts from these cosmids was determined for *Bam*HI and *Kpn* I. The position of seven relevant overlapping cosmids is shown in Fig. 1 along with the composite *Bam*HI and *Kpn* I restriction map of the entire cloned domain. After five rounds of screening, ≈120 kb of contiguous chromosomal DNA was cloned from adriamycin-resistant LZ cells.

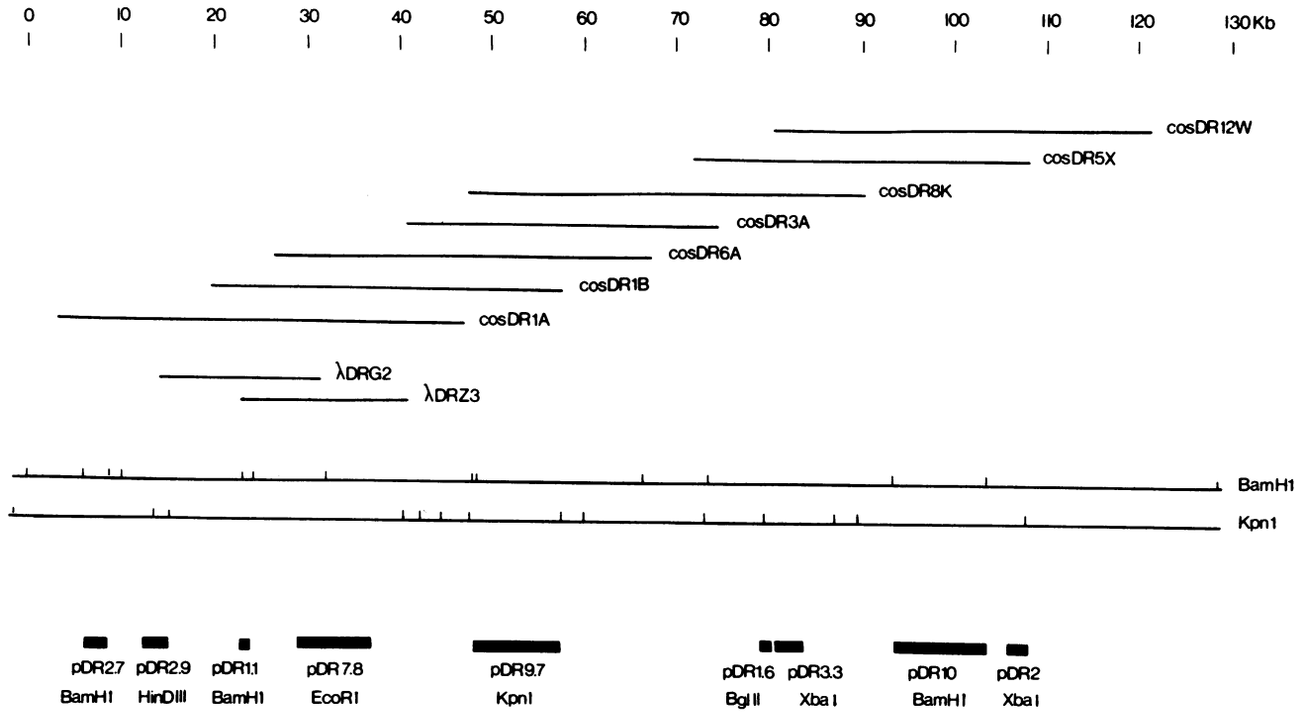

FIG. 1. Map of amplified domain from multidrug-resistant LZ cells. A composite restriction map of cloned domain for restriction endonucleases *Bam*HI and *Kpn* I obtained by analysis of 75 independent clones is presented. The positions of DNA inserts from two representative phage clones and seven overlapping cosmid clones are indicated. The positions of the repeat-free subclones (pDR2.7 to pDR2) used in the hybridization analysis are shown as dark boxes along with the restriction enzyme used for cloning.

Assignment of Cloned Sequences to Fragments of Amplified DNA from LZ Cells. We proceeded to determine (*i*) if the entire cloned region was amplified in LZ DNA and (*ii*) if the set of clones in Fig. 1 constituted an accurate representation of the amplified unit. Our mapping method is based on the in-gel hybridization procedure (12). Briefly, cloned DNA fragments from the cosmids (driver DNA) are used to protect the corresponding ^{32}P-labeled genomic DNA fragments (tracer DNA) from digestion with S1 nuclease following electrophoresis and in-gel renaturation. Two representative examples of such gels are shown in Fig. 2 together with *Bam*HI restriction maps of individual cosmids. In these experiments 10 ng of cosmid DNA digested with *Bam*HI (driver DNA) is mixed with ^{32}P-labeled tracer DNA (see above) and 10 μg of heterologous carrier DNA (human DNA digested with *Eco*RI) prior to electrophoresis. When total LZ DNA digested with *Bam*HI is used both as tracer and driver, the characteristic pattern of amplified DNA fragments is detected over the background bands of repetitive DNA present in the V79/V79 and LZ/LZ lanes (Fig. 2). When *Bam*HI-digested cosmid DNA is used as driver, protection from degradation by S1 nuclease of the homologous genomic DNA fragments is observed in LZ but not in V79 DNA lanes (Fig. 2). In experiments of the type shown in Fig. 2 it was possible to directly assign eight *Bam*HI fragments (present in independent cosmids and spanning 65 kb) to the corresponding set of fragments amplified in LZ cells (LZ tracer/LZ driver lane). This procedure can thus be used for rapid and accurate mapping of DNA amplified to 30–50 copies per mammalian genome.

The above mapping procedure has also facilitated identification of cosmid clones carrying rearranged insert DNA. For example, cosmid 1D (Fig. 2*a*) has five internal *Bam*HI fragments 16, 8, 7.5, 2, and 1.1 kb long. When used as driver in combination with LZ tracer DNA, only three of these segments (16, 8, and 1.1 kb) protected LZ tracer DNA against digestion by S1 nuclease. The two other fragments (7.5 and 2 kb) are therefore rearranged and not representative of the amplified domain in LZ DNA. Another rearranged fragment was identified in cosmid B2 (Fig. 2*b*, 8.5-kb fragment) by the same approach. The correct restriction map of this area was obtained from cosmid B10, which contains contiguous 13-, 1.3-, and 2.7-kb *Bam*HI fragments, all of which hybridize to LZ tracer DNA (Fig. 2*b*).

The mapping approach developed in the present work has two major advantages over conventional analytical techniques: (*i*) the presence of highly repeated interspersed DNA sequences is not an impediment to the analysis; (*ii*) artefacts involving rearrangements of the DNA after insertion in the cloning vector can be readily identified.

It should be noted that a fragment of driver DNA with partial but continuous homology to a genomic (tracer) fragment of the same electrophoretic mobility can protect the latter from S1 nuclease digestion. Examples of this phenomenon are shown in Fig. 2 (cosmid B10, 16-kb *Bam*HI fragment; cosmid 1B, 18-kb *Bam*HI fragment).

Characterization of DNA Sequences Coamplified in LZ and C5 Cells. To determine the portions of the cloned domain that are coamplified in relevant multidrug-resistant cell lines, we performed Southern hybridization with DNA probes isolated from the LZ amplified domain (Fig. 3). The results show that the entire cloned region of ≈120 kb in length is amplified in C5 and LZ cells since probes pDR2.7, pDR2.9, pDR7.8, pDR9.7, pDR10, and pDR2 detect DNA fragments amplified in the LZ and C5 cell lines (Fig. 3). Analyses of Southern blots at different levels of autoradiographic exposure (data not shown) do not indicate the presence of a so-called "gradient of amplification" (25) within the ≈120-kb amplified domain analyzed so far in LZ or C5 cells. Each probe in LZ and C5 DNA also detected a second set of weakly cross-hybridizing

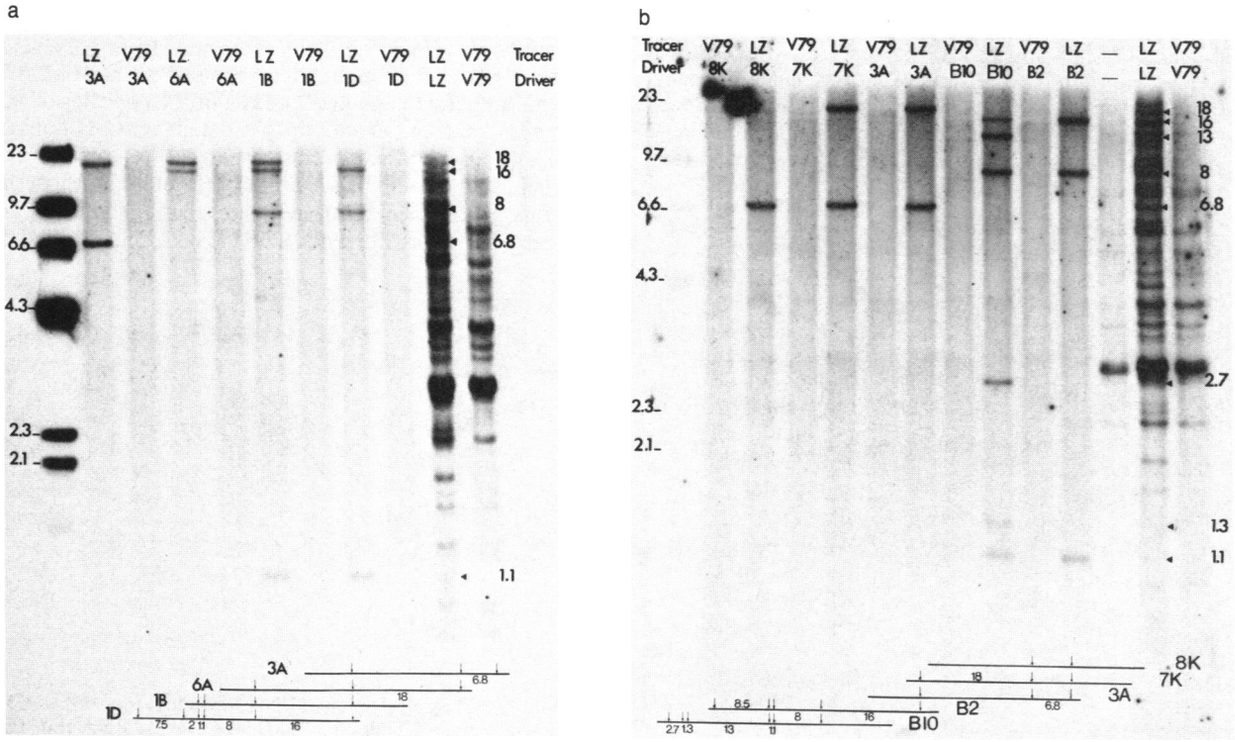

FIG. 2. Assignment by in-gel hybridization of the cloned *Bam*HI fragments to their homologs amplified in LZ cells. Tracer DNA was digested with *Bam*HI and ^{32}P-labeled with T4 DNA polymerase. Genomic driver DNAs were digested with *Bam*HI (LZ and V79 lanes), whereas cosmid driver DNAs were digested with either *Bam*HI alone (*a*) or *Bam*HI together with *Sal* I (*b*). For each lane, both tracer and driver DNAs are identified. The set of *Bam*HI fragments amplified in LZ DNA and contained within the cosmid clones used in this analysis is denoted by arrowheads. The *Bam*HI restriction map of each of the cosmid insert DNAs used is also shown.

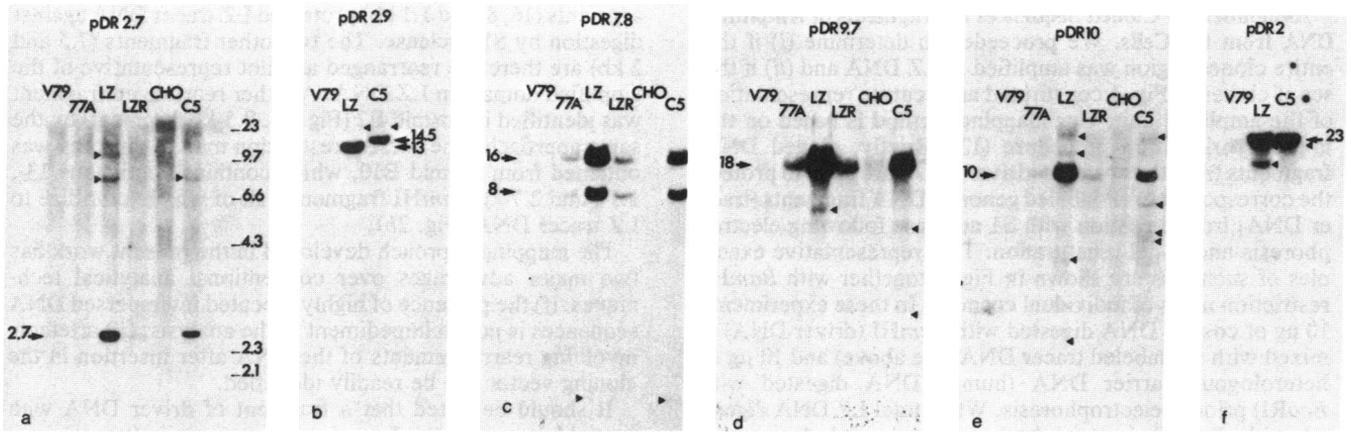

FIG. 3. Characterization of the amplified domain in LZ and C5 cell lines. Southern hybridization analysis of *Bam*HI-digested DNA from V79 and Chinese hamster ovary (CHO) AuxB1 parental cell lines together with their respective multidrug-resistant derivatives 77A, LZ, LZR, and C5. In *a–f* the following ^{32}P-labeled insert DNAs from plasmid subclones overlapping the entire cloned region (Fig. 1) of amplified DNA were used: pDR2.7 (*a*), pDR2.9 (*b*), pDR7.8 (*c*), pDR9.7 (*d*), pDR10 (*e*), pDR2 (*f*). Amplified DNA fragments that exhibit strong hybridization are denoted by arrows with the fragment size in kb. DNA fragments that exhibit weak hybridization are denoted by arrowheads.

fragments, identified in Fig. 3 with small arrows (for instance, in Fig. 3*b*, pDR2.9 detects a 17-kb fragment, and in Fig. 3*e*, pDR10 detects multiple bands). These homologous fragments follow a pattern of amplification similar to the pattern observed for the intensely hybridizing DNA bands from cell lines LZ and C5 (Fig. 4 and data not shown).

Transcription Unit Within the Amplified Domain. To characterize transcriptional products of the amplified DNA region, RNA prepared from V79, 77A, LZ, and C5 cells was hybridized by transfer blotting to three subclones of the cloned DNA (Fig. 5*a*, pDR7.8; Fig. 5*b*, pDR1.6; Fig. 5*c*, pDR2). All three probes detected a large mRNA species transcribed to high levels in the two multidrug-resistant cell lines, C5 and LZ (Fig. 5). Probes mapping to the left of pDR7.8 and to the right of pDR10 did not hybridize to this mRNA species (data not shown). Furthermore, the 77A cells (low level of adriamycin resistance) show a small but significant increase in this mRNA above the very low background level of V79 RNA (Fig. 5*b*). Thus, the region amplified in C5 and LZ cells contains an expressed gene that spans at least 75 kb and encodes a discrete ≈5-kb mRNA species whose intracellular concentration correlates with the relative level of multidrug resistance of each cell line tested. Since cross-hybridization between individual cosmids overlapping this region could not be detected (data not shown), it is most likely that the amplified region contains a single copy of the gene encoding this mRNA rather than a cluster of related sequences.

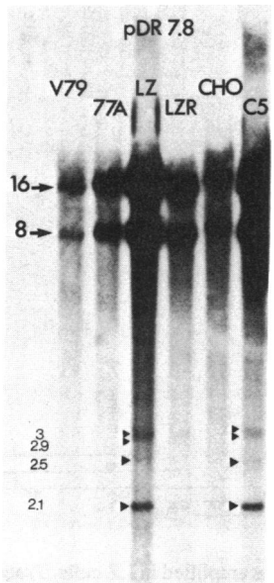

FIG. 4. Amplification of cross-hybridizing sequences in LZ and C5 DNA. This is a 5-fold longer exposure (35 hr) of the autoradiogram shown in Fig. 3*c*.

DISCUSSION

We demonstrate that molecular cloning techniques and in-gel renaturation (12, 13) together provide a powerful and general approach to isolation and characterization of amplified DNA from eukaryotic cells. By using this approach, ≈120 kb of contiguously amplified DNA sequences from LZ cells have been isolated. We found that a gene that spans at least 75 kb of this amplified domain encodes a mRNA species of ≈5 kb, which is abundant in multidrug-resistant cells but not in their drug-sensitive parental counterparts. Preliminary experiments using cloned cDNA probes indicate that transcription of this gene proceeds from a site near the probe pDR7.8 through a site near the probe pDR2 (Fig. 1; unpublished data).

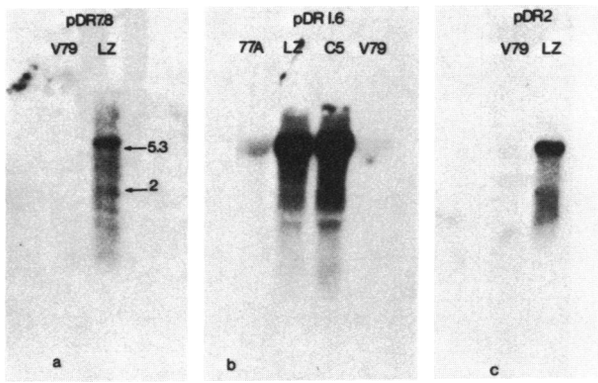

FIG. 5. Characterization of DNA sequences transcribed from the genomic domain amplified in LZ and C5 cells. RNA transfer blot hybridization analysis of total cellular RNA (*b*) from V79, 77A, LZ, and C5 cells and of poly(A)$^+$ RNA (*a* and *c*) from V79 and LZ cells. Twenty micrograms of total cellular RNA (*b*) or 1 μg of poly(A)$^+$ RNA (*a* and *c*) was electrophoresed. Blots were hybridized to ^{32}P-labeled insert DNAs from plasmids pDR7.8 (*a*), pDR1.6 (*b*), and pDR2 (*c*).

The 5-kb mRNA species is likely to play a role in the multidrug-resistant phenotype. The nature of protein(s) encoded by this mRNA and the mechanism of multidrug resistance remain unknown. Ling and co-workers (26, 27) have established a correlation in various cell lines between the degree of colchicine resistance and the increased expression of a 170-kDa membrane glycoprotein, gp170. The length of the mRNA species identified in the present work (≈5 kb) is consistent with it encoding a polypeptide of 170 kDa in size. Furthermore, LZ and 77A cells express levels of gp170 proportional to their respective levels of resistance to adriamycin (unpublished data). On the other hand, Kuo *et al.* (9) have identified a 21-kDa polypeptide that is expressed at high levels in vincristine-resistant hamster cells. Biedler *et al.* have also described a 19-kDa cytosolic protein overexpressed in vincristine-resistant hamster cells (28). Loss of specific 75-kDa and 72-kDa glycoproteins has also been observed in multidrug-resistant cells (29). mRNA and genomic DNA sequences encoding these proteins remain to be identified.

Even though amplification and corresponding overexpression of the gene encoding the 5-kb mRNA are the most likely candidates for a common mechanism of multidrug resistance, other genes may also be involved in the establishment of resistance to a particular cytotoxic agent, in particular, because multidrug-resistant cell lines usually show the highest level of relative drug resistance to the agent used in the original selection process.

In addition to the major set of amplified DNA sequences in LZ DNA, Southern hybridization analyses detected also weakly cross-hybridizing DNA sequences distinct from those in the major set (see Figs. 3 and 4). One interpretation of this result is that the gene present within the amplified domain is a member of a multigene family. A second possibility is that the above minor fragments are the result of DNA rearrangements that have occurred during DNA amplification in LZ cells. Such rearranged DNA sequences have been described in studies of several other amplified genes (30–32). The fact that some of the cross-hybridizing sequences can be detected in the genome of the parental V79 and Chinese hamster ovary AuxB1 cell lines (Fig. 4, 2.1-kb *Bam*HI fragment) argues against this second possibility. Furthermore, preliminary experiments involving cDNA cloning are consistent with the first possibility: we have identified at least two distinct cDNA populations homologous to DNA sequences from the amplified genomic domain (unpublished data).

Our results strongly suggest that the common mechanism of multidrug resistance in the independently derived LZ and C5 cells involves overexpression of the 5-kb mRNA encoded within the amplified domain. Whether the phenotype of multidrug resistance is due to overexpression of the unaltered endogenous copy of the gene or whether alterations in the initial nucleotide sequence (followed by its overexpression) are also essential remains to be determined. Cloned DNA fragments from the genomic domain amplified in the two multidrug-resistant cell lines represent valuable tools to study multidrug resistance in other systems, including human tumors that have developed drug resistance in the course of chemotherapy.

We thank Gail-Lenora Statton and Barbara Doran for secretarial assistance. P.G. is a recipient of a fellowship from the Medical Research Council of Canada. J.C. is supported by a National Institutes of Health training grant (5T 32HL07574). This work was supported by grants from the National Institutes of Health to D.E.H. (CA17575) and A.V. (CA33297) and from the Bristol-Myers Corporation.

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