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Expression of Hamster P-Glycoprotein and Multidrug Resistance in DNA-Mediated Transformants of Mouse LTA Cells

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The overexpression of a plasma membrane glycoprotein, P-glycoprotein, is strongly correlated with the expression of multidrug resistance. This phenotype (frequently observed in cell lines selected for resistance to a single drug) is characterized by cross resistance to many drugs, some of which are used in cancer chemotherapy. In the present study we showed that DNA-mediated transformants of mouse LTA cells with DNA from multidrug-resistant hamster cells acquired the multidrug resistance phenotype, that the transformants contained hamster P-glycoprotein DNA sequences, that these sequences were amplified whereas the recipient mouse P-glycoprotein sequences remained at wild-type levels, and that the overexpressed P-glycoprotein in these cells was of hamster origin. Furthermore, we showed that the hamster P-glycoprotein sequences were transfected independently of a group of genes that were originally coamplified and linked within a 1-megabase-pair region in the donor hamster genome. These data indicate that the high expression of P-glycoprotein is the only alteration required to mediate multidrug resistance.

Cultured cells and transplantable tumors selected for resistance to a single drug can often acquire cross resistance to a wide range of drugs which differ in mode of action, target, and structure (9). This phenotype, called multidrug resistance, parallels the clinical observation that patients whose malignancies recur after primary therapy are often nonresponsive to subsequent combination chemotherapy. A central role for an integral plasma membrane glycoprotein, P-glycoprotein, in mediating multidrug resistance is supported by different observations (9). (i) Independent multidrug-resistant clones selected in our laboratory and those of others consistently overexpress P-glycoprotein (5, 9–12). (ii) Increases in the level of drug resistance are matched by increases in the amount of P-glycoprotein expressed at the cell surface (9–11). (iii) Single-step revertants to wild-type levels of drug resistance show a simultaneous decrease in the level of P-glycoprotein expression to near wild-type levels (10, 11). (iv) P-glycoprotein is highly conserved, and multidrug-resistant tissue culture mutants of hamster, mouse, and human origin all display elevated levels of P-glycoprotein (9–11). (v) The location of P-glycoprotein in the plasma membrane is consistent with a functional role for this molecule in view of the results of drug uptake studies. Such studies have shown that the phenotype is a result of a reduced net intracellular accumulation of drugs (for reviews, see references 2 and 18) and that agents which act on the membrane, such as anaesthetics, nonionic detergents, and calcium channel antagonists, alter the expression of multidrug resistance (for a review, see reference 18).

Despite the close correlation between P-glycoprotein overexpression and multidrug resistance, there is no direct evidence that P-glycoprotein overexpression is the causative molecule in multidrug resistance. We have isolated independent multidrug-resistant transformants of drug-sensitive mouse LTA cells which were transfected with DNA from multidrug-resistant Chinese hamster ovary (CHO) cells. We show that these transformants contain hamster P-glycoprotein sequences which are amplified and that the overexpressed P-glycoprotein detected in these cells is solely of hamster origin. In addition, we show that, of a group of five other linked genes that are coamplified and transcribed at high levels in the donor hamster cell line (22), none was cotransfected. From these data, we conclude that P-glycoprotein overexpression alone can induce multidrug resistance.

MATERIALS AND METHODS

Cell culture. LTA is an adenine phosphoribosyl transferase-deficient mouse fibroblast cell line derived from LMTK$^-$ cells, a thymidine kinase-deficient cell line (5, 13). ECH^R is a colchicine-resistant mutant isolated from an ethyl methanesulfonate-treated LMTK$^-$ culture (5). AuxB1 is a glycine-adenosine-thymidine auxotroph CHO line (16). It is the parent of the colchicine-resistant line CH^{R}C5, which was selected in three steps for colchicine resistance with ethyl methanesulfonate mutagenesis (14). CCRF-CEM is a human leukemic line (3). All transformants (LC5B1, LC5B3, LC5A10, and LC5A11) were isolated from cultures of LTA cells exposed to calcium phosphate-precipitated CH^{R}C5 genomic DNA. All cell lines were maintained in αMEM (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. Cultures were grown as monolayers at 37°C in humidified air containing 5% CO_2. Resistance values were determined as described previously (1) and are expressed as a ratio of the level of resistance of a cell line relative to its parent. For the transformants, the parent line

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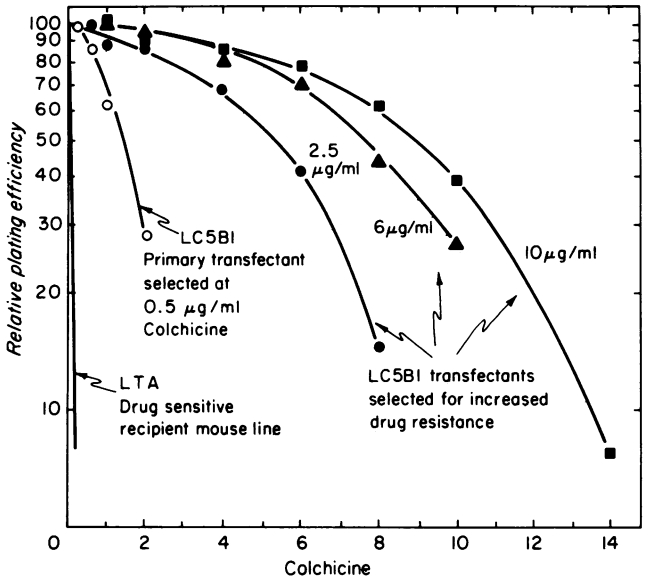

FIG. 1. Relative plating efficiencies of a transformant at various levels of resistance and of the mouse recipient. The relative plating efficiency is expressed as a percentage of the plating efficiency of a cell line plated in a drug relative to that of the same line plated without a drug. Shown are the relative plating efficiencies of the primary transformant LC5B1 selected at 0.5 μg ml^{-1} and those of LC5B1 selected in continuous culture for increased resistance (2.5, 6.0 and 10.0 μg ml^{-1}, respectively). Also shown is the relative plating efficiency of LTA, the drug-sensitive recipient mouse line. Colchicine measured in micrograms per milliliter.

is considered to be the recipient, LTA. The number in parentheses after the name of a cell line denotes the level of colchicine (μg ml^{-1}) at which the cells were selected and grown.

DNA-mediated transformation. Mouse LTA cells were transformed with genomic DNA by the calcium phosphate precipitation technique (5, 19). Twenty micrograms of genomic DNA precipitate was applied to each 100-mm tissue culture dish containing an overnight culture of recipient cells seeded at 7×10^5 cells per dish. Absorption was continued at 37°C for 24 h, after which the cells were treated with dimethyl sulfoxide (final concentration 6.5%) for 30 min at 37°C. This medium was removed and replaced with fresh medium for 24 h before selective conditions were applied. Selection for transformants involved a relatively stringent three-step regimen of 0.1 μg of colchicine ml^{-1} for 3 days, followed by 0.3 μg of colchicine ml^{-1} for 3 days, and finally 0.5 or 1.0 μg of colchicine ml^{-1} for 2 to 3 weeks. This protocol has been shown to yield a low frequency (3×10^{-8}) of spontaneous drug-resistant mutants (5). Surviving colonies were transfered by being scraped and aspirated with a sterile Pasteur pipette into growth medium containing colchicine at the final selection concentration for growth to bulk culture.

Selection for higher levels of colchicine resistance was achieved by stepwise increases in drug concentrations during continuous culture. T_{75} tissue culture flasks containing different concentrations of the drug were seeded with approximately 2×10^5 cells. The flask with the highest drug concentration showing significant cell growth after 2 weeks was used for further selection. Cells from this flask were grown to bulk culture with the drug over a 2- to 3-week period, and a portion of these cells was frozen. The cells were subjected to several rounds of this selection protocol; for example, the transformant LC5B1 (0.5), initially resistant to 0.5 μg of colchicine ml^{-1}, was selected for increased drug resistance levels of 2.5, 6.0, and 10.0 μg of colchicine ml^{-1}, respectively. The level of colchicine resistance of LC5B1 at each selection step is shown in Fig. 1.

The transformants were independently derived. Clones LC5B1 and LC5B3 were obtained from the same experiment but from different plates. Clones LC5A10 and LC5A11 were derived from another experiment with a different preparation of CH^RC5 DNA and were also from different plates. In a separate control experiment, in which recipient cells received either no DNA or DNA from wild-type mouse (LTA), hamster (AuxB1), or human (CCRF-CEM) cells, only one resistant colony was isolated, for an overall frequency of $\sim 10^{-8}$. This colony, which gave rise to the line LC1, was from a control plate which received no DNA. LC1 was initially picked and grown in 0.3 μg ml^{-1} and then was selected for increasing resistance as described above to yield LC1(1.0) and ultimately LC1(10.0).

Southern blot analysis. DNA samples from all cell lines were isolated from a guanidine thiocyanate cesium chloride gradient (4) with modifications (7), dialyzed against TE (1 mM Tris [pH 8.0], 0.1 mM EDTA), extracted with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1), precipitated with 0.3 M sodium acetate and 3 volumes of ethanol, and suspended in water. After digestion with the appropriate restriction enzyme (Boehringer GmbH, Mannheim, Federal Republic of Germany), equivalent amounts of DNA were elecrophoresed in 0.5 or 0.6% agarose gels in TBE (0.089 M Tris, 0.092 M boric acid, 0.002 M EDTA [pH 8.0]) as judged by A_{260} and ethidium bromide staining intensity of aliquots of digested DNA electrophoresed in test gels. Lambda DNAs digested with *Hin*dIII and *Eco*RI were coelectrophoresed to serve as molecular weight markers. Probes were nick translated (15) to a specific activity of approximately 2×10^8 cpm μg^{-1}. Probe pCHP1, a 640-base-pair cDNA fragment of P-glycoprotein, was isolated from a λgt11 colchicine-resistant CHO cDNA library (17). The remaining probes (cp16, cp6, cp19, cp30, and cp34) were isolated from a cDNA library of CH^RC5 by differential screening with CH^RC5- and AuxB1-labeled cDNA and represent distinct genes that are amplified and overexpressed in CH^RC5 cells (22). Autoradiograms were obtained by exposing filters at −70°C to X-AR5 film (Eastman Kodak Co., Rochester, N.Y.) with the use of Cronex intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

The Southern blots described in Fig. 2a and b were done with Zetabind paper (AMF Cuno, Meriden, Conn.), and gels were prepared, transferred, prehybridized, and hybridized essentially as described by the manufacturer. No acid hydrolysis of the gel was carried out. Filters were hybridized at 42°C with 10% dextran sulfate and 10^6 cpm of probe pCHP1 ml^{-1}. Washes were done as described by AMF Cuno, with a final wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 60°C. Southern blots onto nitrocellulose (BA85; Schleicher & Schuell, Inc., N.H.) (summarized in Table 2) were done by acid degradation of the gel (0.25 M HCl, 20 min at room temperature, followed by denaturation, neutralization, and capillary transfer in 20× SSC by standard procedures (20). Filters were baked 2 h under vacuum at 80°C and prehybridized for 2 h at 65°C in 6× SSC–0.1% sodium dodecyl sulfate–0.02% polyvinyl pyrrolidone–0.02% bovine serum

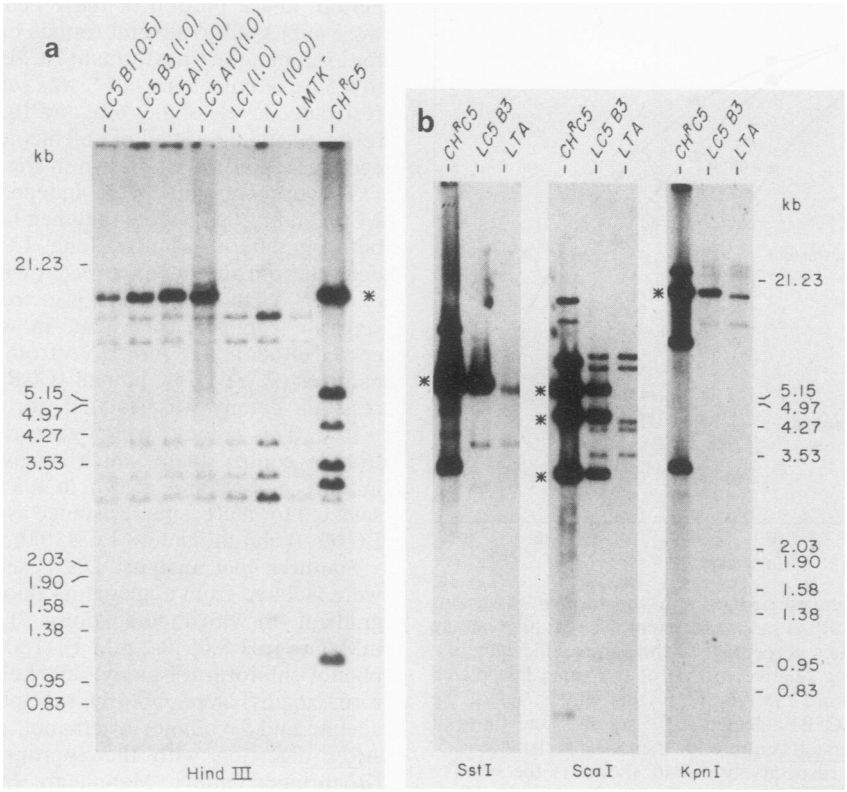

FIG. 2. Southern blot analysis of transformants with the P-glycoprotein cDNA probe pCHP1. Six micrograms of DNA per lane was electrophoresed in agarose gels, transferred to Zetabind paper, and probed with pCHP1 as described in Materials and Methods. (a) Comparison of *Hin*dIII digest of the four independent transformants LC5B1, LC5B3, LC5A10, and LC5A11 with similar digests of the donor line CH^RC5 and of the wild-type mouse pattern of $LMTK^-$. The pCHP1-homologous *Hin*dIII restriction pattern of $LMTK^-$ is identical to that of LTA, the drug-sensitive mouse recipient in the transformation experiments. Also shown is a *Hin*dIII digest of the spontaneous mouse cell mutant LC1 at two levels of resistance (1.0 and 10.0 μg ml^{-1}, respectively). Bands in CH^RC5 were amplified compared with its parent line, AuxB1 (data not shown [17]). The $LMTK^-$ lane was slightly underloaded in this experiment. (b) Comparison of one of the transformants, LC5B3, with donor (CH^RC5) and recipient (LTA) cell lines. DNA samples were digested with either *Sst*I, *Sca*I, or *Kpn*I as indicated below each gel. The patterns of the remaining three transformants are identical to that of LC5B3 (data not shown). Asterisks indicate bands in CH^RC5, for which a band of apparently identical mobility is found in the transformants. Sizes in kilobases (kb) of coelectrophoresed λ DNA digested with *Hin*dIII and *Eco*RI are indicated.

albumin–0.02% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.)–50 μg of denatured salmon testes DNA ml^{-1}. Hybridization was carried out in the same mixture for 40 h at 65°C with the addition of 10^6 cpm of probe pCHP1, cp16, cp6, cp19, cp30, or cp34 ml^{-1}. Nitrocellulose filters were washed first in 3× SSC for 5 h at 65°C and then in 0.1× SSC for 1 h at 65°C.

Western blot analysis. Immunoblot (Western blot) analysis of membrane proteins was undertaken as described previously (11, 12). Sixty micrograms of membrane protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Fairbanks et al. (8) and transferred by electroblotting to nitrocellulose by the method of Towbin et al. (21). Blots were blocked in 3% bovine serum albumin in Tris-saline with sodium azide (10.5 mM Tris hydrochloride [pH 7.4], 175 mM NaCl, 15 mM NaN_3) overnight at 37°C and were then incubated in fresh blocking buffer for 16 h with shaking at 4°C with 5×10^5 cpm of ^{125}I-labeled antibody ml^{-1} radioiodinated by the chloramine T method (5). Blots were then washed for 3 h at room temperature in six changes of phosphate-buffered saline. Autoradiograms were obtained as described above for Southern blots.

RESULTS

Description of transformants. An important concern for the isolation of transformants was that the selection strategy would be stringent enough to minimize the possibility of isolating spontaneous mouse cell mutants while allowing the expression of the transformed phenotype. These criteria were satisfied by a three-step selection protocol with dose increases at 3-day intervals (5). This protocol yielded colchicine-resistant colonies at a frequency of approximately 100 times that of spontaneous mutants. Colchicine-resistant transformants were tested for the expression of multidrug resistance. The cross-resistance profiles of two independent transformants, LC5B1 and LC5A11, are shown in Table 1, as well as those of the donor line CH^RC5 and its parent (AuxB1), the recipient line (LTA), and the mouse mutants ECH^R and LC1. Both transformants display the full complexity of the multidrug resistance phenotype, shown by cross resistance to vincristine and adriamycin and by collateral sensitivity to acronycine. The transformants LC5B3 and LC5A10 expressed patterns of drug resistance and collateral sensitivity similar to those of LC5B1 and LC5A11 (data not shown). Although the transformants expressed profiles of

resistance and collateral sensitivity which resemble that of the donor cell line, CH^RC5, consistent with the expression of hamster genes, the mouse cell mutant LC1 also displayed a similar drug resistance and sensitivity profile. Thus it is not possible to determine on this basis whether these transformants express hamster or mouse multidrug resistance genes.

Detection of hamster P-glycoprotein sequences in transformants. The cDNA clone pCHP1 is a 640-base-pair fragment of a P-glycoprotein hamster cDNA. It was isolated from a λgt11 cDNA library of a colchicine-resistant CHO cell line with a P-glycoprotein-specific monoclonal antibody (17). It can cross-hybridize to both mouse and human DNA under stringent conditions. Because hamster and mouse pCHP1-homologous sequences can be distinguished by differences in restriction patterns, pCHP1 was used to determine whether the transformants had taken up hamster P-glycoprotein sequences. Fig. 2a and b are autoradiograms of pCHP1-probed Southern blots of transformant DNAs compared with the donor cell line (CH^RC5) and with drug-sensitive and -resistant mouse cell lines ($LMTK^-$ and LC1, respectively). None of the enzymes used in these two figure panels cut within pCHP1. Figure 2a consists of *Hin*dIII digests of the four transformants, compared with the hamster and mouse cell patterns. In all the multidrug-resistant hamster and mouse cell lines, amplification of P-glycoprotein sequences can be observed, the level of which correlates with the level of drug resistance. The band pattern of the hamster cell line CH^RC5 is clearly different from that of each of the mouse cell lines. In all the transformants, a new, high-molecular-weight band can be seen, apparently identical in mobility to one of the seven bands found in the donor cell line, CH^RC5 (marked by an asterisk). It is interesting to note that all four independent transformants contain the same presumptive hamster cell band. In addition, it is apparent that only this hamster cell band was amplified in the transformants; the mouse cell bands remained at wild-type levels.

Representative digests of one of the clones, LC5B3, with the enzymes *Sst*I, *Sca*I, and *Kpn*I and equivalent digests of CH^RC5 and LTA DNA are shown in Fig. 2b. In each case, the transformant contains only a subset of the donor hamster P-glycoprotein sequences. The mouse P-glycoprotein sequences remain at wild-type levels, whereas the transfected hamster sequences have become amplified. The other three transformants contained the same subset of hamster cell bands as in LC5B3 (data not shown).

It cannot yet be determined whether the single hamster cell band in the transformants observed in digests with *Hin*dIII, *Sst*I, and *Kpn*I represents one gene or more than one gene with a restriction fragment in common. The three *Sca*I hamster cell bands may indicate three genes or may indicate that the genomic sequences contained in the cDNA probe pCHP1 are separated by at least two *Sca*I site-containing introns; pCHP1 itself contains no *Sca*I sites.

TABLE 1. Drug resistance profiles of cell lines

Cell line	Relative resistance[a]			
	Colchicine	Vincristine	Adriamycin	Acronycine
Mouse				
LTA	1	1	1	1
ECH^R(10.0)	200	75	83	~1.0
LC1(1.0)	22	17	10	0.9
LC1(10.0)	216	83	83	0.09
Hamster				
AuxB1	1	1	1	1
CH^RC5	150	38	43	0.1
Transformant				
LC5B1(0.5)	33	25	33	1.0
LC5B1(6.0)	183	133	183	0.15
LC5A11(1.0)	66	50	33	~1.0
LC5A11(6.0)	200	133	≥165	0.08

[a] LTA is the parent of the resistant mouse lines, and its D_{10}s for colchicine, vincristine, adriamycin, and acronycine were 0.06, 0.06, 0.06, and 7 μg ml^{-1}, respectively. (D_{10} is the drug concentration that reduced the relative plating efficiency by 90%.) The D_{10}s for AuxB1, the parent of CH^RC5, were 0.04, 0.04, 0.07, and ~20 μg ml^{-1}, respectively. Relative resistance for the transformants was calculated with respect to the D_{10}s of LTA.

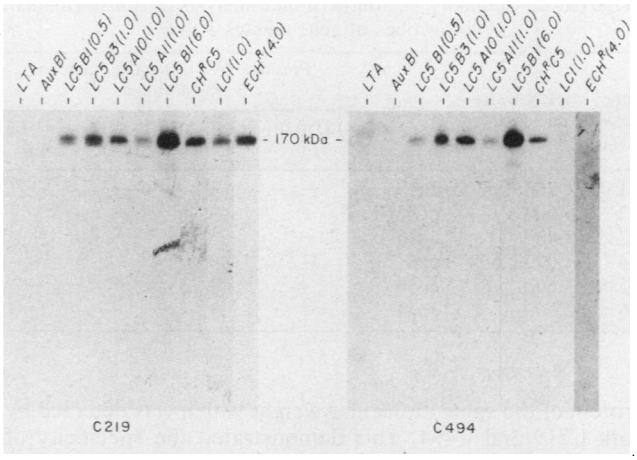

FIG. 3. Western blot analysis of plasma membrane preparations with the monoclonal antibodies C219 (which recognizes hamster, human, and mouse P-glycoproteins) and C494 (which recognizes hamster and human but not mouse P-glycoproteins). Duplicate samples of membrane proteins (60 μg) were separated on polyacrylamide gels, transferred to nitrocellulose, and probed with ^{125}I-labeled C219 or C494 antibodies as described in Materials and Methods. Exposures were for 21 h for C219-probed blots and the C494-probed ECH^R blot and for 2 h for the remaining C494-probed samples. All samples probed with the same antibody were run on the same gel, except the ECH^R samples, which were processed separately. kDa, Kilodaltons.

Western blot analysis. Having shown that hamster P-glycoprotein sequences are present and preferentially amplified in the transformants, it was important to determine whether the protein encoded by these sequences was expressed. It was originally shown with polyclonal antibodies that all the transformants possess high levels of P-glycoprotein in their plasma membranes (5); however, no distinction between P-glycoprotein of hamster or mouse origin could be made at that time. Recently, monoclonal antibodies specific for P-glycoprotein have been obtained (10). One class of monoclonal antibodies, represented here by C219, recognizes an epitope conserved among hamster, mouse, and human P-glycoproteins. Another class of antibody, represented by C494, recognizes a separate epitope shared between hamster and human but not mouse P-glycoproteins. Western blots of duplicate gels were therefore probed with either C219 or C494 ^{125}I-labeled monoclonal antibodies (Fig. 3). The multidrug-resistant mouse mutants ECH^R and LC1 both expressed a 170-kilodalton protein that was detected by C219 but not by C494. CH^RC5, the hamster cell mutant and donor for the transformants, expressed a

TABLE 2. Summary of Southern blot analysis of transformants with probes of gene classes 1 to 6

Gene class	mRNA size (nucleotides)	Probe	Presence (+) of hamster gene in transformant			
			LC5A10 (1.0)	LC5A11 (1.0)	LC5B1 (0.5)	LC5B3 (1.0)
1	750	cp19	−	−	−	−
2	4,500	pCHP1	+	+	+	+
3	3,400	cp16	−	−	−	−
4	1,000/2,500	cp6	−	−	−	−
5	3,600	cp30	−	−	−	−
6	2,600	cp34	−	−	−	−

protein of the same molecular weight which was detected by both C219 and C494. This demonstrated the specificity of C494 for hamster P-glycoprotein. The P-glycoprotein expressed in the transformants was recognized by both C219 and C494, which indicated that the P-glycoprotein detected in the transformants was a product of the transfected hamster sequences. These data, combined with the results described above from Southern blot analysis, indicated that the transformants acquired the multidrug resistance phenotype as a result of the uptake and overexpression of donor P-glycoprotein sequences.

Southern blot analysis of the transformants with probes from the CH^{R}C5 amplicon. A series of cDNA clones have been isolated from overexpressed CH^{R}C5 mRNAs by differential screening (22). These clones were grouped into six classes by the size of transcript(s) recognized in RNA blots and the absence of cross-hybridization with each other. The genomic sequences of each of these cDNAs were amplified in CH^{R}C5. The class 6 gene, which has not been previously described, was also identified by differential screening of CH^{R}C5 cDNA (22). A 2,600-nucleotide mRNA that is recognized by the 660-base-pair class 6 cDNA, cp34, is overexpressed in some multidrug-resistant Chinese hamster lung cell lines in a pattern similar to those of classes 4 and 5 (6) (data not shown). One class of clones, class 2, has been shown by cross-hybridization to pCHP1 to encode P-glycoprotein. Class 4 has been found to encode the cytoplasmic protein, sorcin/V19, which is overexpressed in some multidrug-resistant cell lines (A. M. Van der Bliek, M. B. Meyers, J. L. Biedler, E. Hes, and P. Borst, EMBO J., in press). Linkage analysis by pulsed-field gradient gel electrophoresis (22) and by in situ hybridization with cDNAs of classes 2, 4, and 5 (A. P. M. Jongsma, B. A. Spengler, A. M. Van der Bliek, P. Borst, and J. L. Biedler, submitted for publication) has established that all six genes are part of one large amplicon. Coamplification of class 2, 4, 5, and 6 genes has also been detected in multidrug-resistant human and mouse cell lines (2780 AD and ECH^R, respectively; data not shown).

Southern blot analysis of the transformants with probes of each class was undertaken as a means of testing whether transfection and amplification of any of the linked genes are required for multidrug resistance. The data from this experiment are shown in Table 2. With interspecies restriction-fragment-length polymorphisms, it could be determined that of the six linked hamster genes, only P-glycoprotein sequences were detectable in the transformants; in addition, these were amplified as shown above. These data indicate that the amplification of the genes of classes 1 and 3 to 6 observed in a number of multidrug-resistant cell lines was probably coincidental, contingent upon the linkage of these genes to the P-glycoprotein genes. This supports the conclusion of de Bruijn et al. (6). They found that the P-glycoprotein-encoding class 2 genes were the only ones consistently amplified in three independent multidrug-resistant cell lines, each selected with vincristine, daunorubicin, or actinomycin D. In addition, these results indicate that P-glycoprotein was transfected free of closely linked genes, which is further evidence that the transfection and resultant overexpression of P-glycoprotein alone can mediate multidrug resistance.

DISCUSSION

We previously reported a reproducible DNA-mediated transformation protocol in which recipient drug-sensitive mouse cells acquired the multidrug resistant phenotype when transfected with DNA from multidrug-resistant CHO cells (5). We showed that (i) a difference exists between resistant and wild-type CHO donor DNAs in their ability to confer the multidrug resistance phenotype onto transformants, (ii) unlinked markers were not cotransfected, and (iii) the transformants all overexpressed P-glycoprotein concurrent with the expression of multidrug resistance. These observations are consistent with the transfection of the P-glycoprotein gene or genes and their central role in multidrug resistance. However, it was not possible at that time to demonstrate that the P-glycoprotein expressed in the transformants was of hamster origin, because the P-glycoprotein-specific polyclonal serum was unable to distinguish between hamster and mouse P-glycoprotein. There remained the possibility that some other gene or genes had been transfected, which in turn induced overexpression of mouse P-glycoprotein. In this paper we show that (i) based on restriction patterns of pCHP1-homologous sequences, all the transformants took up donor P-glycoprotein sequences and these were preferentially amplified over mouse P-glycoprotein sequences; (ii) the amplified hamster sequences are expressed as an intact 170-kilodalton P-glycoprotein, which can be distinguished from mouse P-glycoprotein by a monoclonal antibody; and (iii) the transfected P-glycoprotein sequences are not accompanied by genes which are closely linked to P-glycoprotein in the donor cell and which are amplified and transcribed at high levels in the donor cell. On the basis of these observations, we conclude that it is the overexpression of P-glycoproteins alone that is responsible for multidrug resistance. This does not rule out the possibility that other gene products modulate the phenotype (presumably these products would function at wild-type levels), but we have clearly demonstrated that P-glycoprotein overexpression is the alteration that induces multidrug resistance. Other changes detected in multidrug-resistant cells could be ancillary responses to this change in membrane structure.

We have proposed that P-glycoprotein is encoded by a multigene family, based on the complex band patterns observed with Southern blot analysis of *Eco*RI-digested genomic DNA probed with pCHP1 (17). Multiple bands were again observed with the four restriction enzymes used here, supporting the multigene family hypothesis. This family is thought to be linked, because in the single-step, multidrug-resistant CHO mutant, CH^{R}A3, a simultaneous amplification of all the pCHP1-homologous bands was detected (17). Furthermore, pulsed-field gradient electrophoresis revealed only one linkage group carrying P-glycoprotein sequences (22). de Bruijn et al. (6) have suggested that there are at least two or three members in this putative multigene family,

based on differential amplification detected in restriction bands patterns. Two observations are significant to the discussion of the P-glycoprotein multigene family. The first is that the four transformants contain a subset of the donor complement of bands. Since all the transformants express functional, full-length P-glycoprotein, this is further proof that the multiple bands detected in the donor DNA are indicative of a multigene family. We can also conclude that less than the full complement of P-glycoprotein genes is required for expression of multidrug resistance in the transformants, a conclusion that is consistent with the data of Gros et al. (9a), who have shown that the transfection of a full-length cDNA of a single presumptive P-glycoprotein gene can result in multidrug-resistant transformants. At this time, we cannot determine how many hamster P-glycoprotein genes are contained and expressed in the transformants. The second observation relevant to the discussion of the P-glycoprotein multigene family is that the transformants contain not only a subset of the donor complement of bands but also the very same subset of bands. One explanation is that these bands represent the only functional member(s) of the P-glycoprotein multigene family. Alternatively, it is possible that the transformation process somehow favors this particular subset. For example, it is possible that the transfected hamster DNA contains an origin of replication, which would render this subset more easily amplifiable after transformation than would DNA containing other P-glycoprotein genes. Regardless of the explanation, a subset of the P-glycoprotein multigene family has been identified which is able to mediate multidrug resistance.

We do not know what role a P-glycoprotein multigene family plays in the multidrug resistance phenotype. The complexity of this phenotype may reflect a synergism of the individual functions of each member. Differential expression of the members may result in the variability in cross resistance profiles between multidrug-resistant cell lines. We are currently characterizing cDNA and genomic clones representing each family member. From these we can generate gene-specific probes that can be used to address the above-mentioned questions and to explore the tissue-specific and temporal expression of the P-glycoprotein multigene family in the developing and adult organism.

The above-described transformation experiments identify a subset of the P-glycoprotein multigene family, which can be expressed and which can function in mouse cells. This ubiquity of expression and function across a species barrier indicates evolutionary conservation and further suggests an important role for P-glycoprotein in the normal cell. Extensive homology of a partial P-glycoprotein amino acid sequence to the bacterial transport protein, hemolysin B, dramatically emphasizes the evolutionary conservation of the molecule (8a). On the basis of this homology and homology to a number of other bacterial transport proteins, we have proposed that P-glycoprotein may act as an energy-dependent efflux pump. We can only speculate as to the nature of the molecules whose transport is controlled by P-glycoprotein in the normal cell. Further analysis of P-glycoprotein, including its structure and the role of the multigene family, is expected to help understanding of both the regulation of membrane transport in normal cells and the problem of tumor cell resistance to chemotherapy.

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