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Cloning mammalian genes by expression selection of genetic suppressor elements: Association of kinesin with drug resistance and cell immortalization

(cDNA normalization/retroviral vectors/antisense RNA/senescence/etoposide)

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ABSTRACT **We describe a general strategy for cloning mammalian genes whose downregulation results in a selectable phenotype. This strategy is based on expression selection of genetic suppressor elements (GSEs), cDNA fragments encoding either specific peptides that act as dominant inhibitors of protein function or antisense RNA segments that efficiently inhibit gene expression. Since GSEs counteract the gene from which they are derived, they can be used as dominant selectable markers for the phenotype associated with downregulation of the corresponding gene. A retroviral library containing random fragments of normalized (uniform abundance) cDNA expressed in mouse NIH 3T3 cells was used to select for GSEs inducing resistance to the anticancer drug etoposide. Three GSEs were isolated, two of which are derived from unknown genes and the third encodes antisense RNA for the heavy chain of a motor protein kinesin. The kinesin-derived GSE induces resistance to several DNA-damaging drugs and immortalizes senescent mouse embryo fibroblasts, indicating that kinesin is involved in the mechanisms of drug sensitivity and *in vitro* senescence. Expression of the human kinesin heavy-chain gene was decreased in four of four etoposide-resistant HeLa cell lines, derived by conventional drug selection, indicating that downregulation of kinesin represents a natural mechanism of drug resistance in mammalian cells.**

Expression selection has been often used to clone mammalian genes whose mutation or overexpression results in a dominant selectable phenotype. Many phenotypes, however, stem from downregulation rather than overexpression or dominant mutations of specific genes. Expression selection of cDNAs cloned in inverse orientation and encoding antisense RNAs has been proposed as a general approach to cloning such genes (1). The utility of this strategy, however, is limited by the fact that not all genes can be efficiently suppressed by antisense RNA (2). In addition, genes expressed at low levels are underrepresented in both conventional and antisense cDNA libraries, and their isolation is further complicated by the limited efficacy of DNA transfection used in standard expression selection protocols.

We now describe a comprehensive strategy for cloning mammalian genes whose downregulation results in a selectable phenotype. This strategy is based on expression selection of genetic suppressor elements (GSEs), cDNA fragments encoding either specific peptides that act as dominant inhibitors of protein function or antisense RNA segments that are efficient in inhibition of gene expression (3). Since the GSEs counteract the gene from which they are derived, they can be used as dominant selectable markers for the phenotype associated with downregulation of the corresponding gene. We have previously described the procedures for selecting sense-oriented (peptide encoding) and antisense-oriented GSEs in bacterial and mammalian cells by using expression libraries carrying random 200- to 500-bp fragments of bacteriophage λ DNA (3) or of cloned cDNA of human topoisomerase II (topo II) (4). We have now expanded this approach to isolate GSEs derived from unknown genes by using an expression library containing random cDNA fragments of virtually all genes expressed in a mammalian cell. To enable the isolation of GSEs from genes expressed at low levels, random fragments of total cellular cDNA are subjected to normalization, a procedure that equalizes the representation of differentially expressed mRNA sequences in a cDNA preparation (5, 6). In addition, the cDNA library is introduced into the recipient cells by retroviral transduction rather than DNA transfection. This allows us to maximize the transfer efficiency and to simplify the isolation of GSEs from the selected cells, which acquire only a small number of integrated proviral inserts.

In the present study, we have used this strategy to identify genes that potentiate the cytostatic or cytotoxic response to anticancer drugs. The products of these drug-sensitivity genes may function in drug uptake, prodrug activation, target recognition, or induction of growth arrest or programmed cell death in response to drug-induced lesions (7). We have previously shown that GSEs derived from cloned cDNA of one such gene, topo II, induce resistance to etoposide and other topo II-interactive agents (4). We have now used etoposide selection to isolate GSEs from a normalized cDNA fragment library of mouse NIH 3T3 cells to identify additional genes associated with etoposide sensitivity.†

MATERIALS AND METHODS

Preparation of a Normalized cDNA Library. Poly(A)$^+$ RNA from NIH 3T3 cells was fragmented by boiling for 5 min. Double-stranded cDNA was synthesized by using random hexanucleotide primers (8) and a cDNA synthesis system (GIBCO). cDNA was ligated with an adaptor, prepared by annealing two complementary synthetic oligodeoxyribonucleotides: 5′-AATCATCGATGGATGGATGG-3′ (ATGS) and 5′-CCATCCATCCATCGATGATTAAA-3′ (ATGA). After ligation, cDNA was size-fractionated by polyacrylamide gel electrophoresis, and gel-purified 200- to 500-bp fragments were amplified by PCR using ATGS as a PCR primer. PCR

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Abbreviations: GSE, genetic suppressor element; topo II, topoisomerase II; MEF, mouse embryo fibroblast.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L27153, L27154, L27155, and L29223).

was carried out in 12 separate reactions that were subsequently combined. For normalization (6), PCR-amplified cDNA at 400 μg/ml was heat-denatured and allowed to reanneal for 24, 48, 72, or 100 h. Single-stranded and double-stranded DNA from each time point was separated by hydroxyapatite chromatography. Each cDNA fraction was reamplified by PCR and analyzed by parallel Southern hybridizations with different cDNA probes. The single-stranded cDNA fraction obtained after 100 h of reannealing was cloned into the *Cla* I site of the retroviral plasmid vector pLNCX (9). The resulting library contained ≈5 × 10^7 clones, >60% of which contained inserts.

Library Transduction and Etoposide Selection. A mixture of 3 × 10^6 ecotropic and 3 × 10^6 amphotropic packaging cells (10) was transfected with 100 μg of library DNA or insert-free pLNCX plasmid (control) by a standard calcium phosphate procedure, and the culture was incubated for 10 days in the presence of Polybrene (4 μg/ml) to allow the virus to spread. The resulting cell population was 100% G418 resistant, contained multiple integrated proviruses in each cell (according to Southern hybridization), and produced >10^6 infectious particles per ml (as estimated by G418 selection of infected NIH 3T3 cells). The uniformity of sequence representation in the library-derived retroviral population was monitored by DNA extraction from NIH 3T3 cells infected with the virus-containing supernatant, followed by PCR amplification of inserts using ATGS oligonucleotide as a primer, and Southern hybridization of PCR-amplified DNA with actin, tubulin, and c-*myc* probes.

The infected mixture of packaging cell lines was exposed to etoposide (350 ng/ml) for 15 days and then allowed to grow without drug for 2 more weeks. The virus present in the supernatant of the surviving library-transduced cells was then used to infect NIH 3T3 cells, followed by etoposide selection. DNA from the etoposide-selected NIH 3T3 cells was used for PCR amplification and recloning of proviral inserts by a modification of the previously described procedure (4). The following oligonucleotides were used as PCR primers: 5′-CCAAGCTTTGTTTACATCGATGGATG-3′ (sense) and 5′-ATGGCGTTAACTTAAGCTAGCTTGC-CAAACCTAC-3′ (antisense). The PCR products were digested with *Cla* I and *Hpa* I and cloned into the corresponding cloning sites of LNCX in the same orientation as in the original clones.

Identification of GSEs Conferring Etoposide Resistance. A total of 42 clones, obtained after two independent selection experiments, were tested in batches or individually for the ability to confer etoposide resistance after retroviral transduction into NIH 3T3 cells. For testing, NIH 3T3 cells (10^5 cells per 100-mm plate) were infected several times with individual retroviruses until all the cells carried a provirus (as determined by G418 selection on parallel plates). Cell survival was determined after growth in several different concentrations of etoposide for 4–7 days, followed by 1–2 weeks in drug-free medium. Four clones, three of them nonidentical, were found to induce etoposide resistance. These clones were sequenced and analyzed for homology to nucleic acid and protein sequences present in the National Center for Biotechnology Information data base, using the BLASTN and BLASTX network programs for homology search (11).

Cloning of *Khcs* cDNA. The anti-Khcs GSE clone was used as a probe to screen 400,000 clones of a λ gt10 cDNA library prepared from the RNA of mouse BALB/c 3T3 cells synchronized at $G_0 \rightarrow G_1$ transition (12). Two hybridizing clones were purified, sequenced, and found to contain overlapping cDNA inserts incorporating the sequence corresponding to anti-Khcs (with a single nucleotide mismatch). The sequence corresponding to the 5′ end of *Khcs* mRNA was isolated by "anchored PCR" (13, 14), using the 5′-Race kit (GIBCO). The *Khcs*-specific antisense primers, used in two consecutive anchored PCRs, were 5′-CATGAAGTTACCCTCCATCG-3′ and 5′-GCACTATTGACTTGGATAAT-3′.

Drug Resistance and Immortalization Assays. For drug resistance assays, NIH 3T3 cells were infected 10 times with ecotropic virus derived from the LNCX vector without an insert or with the anti-Khcs GSE. The virus titers were >10^5 per ml. Under these conditions, all the cells were infected and contained more than one copy of integrated provirus per cell. For growth inhibition assays, infected cells were plated in 12-well plates at 10^4 cells per well and exposed to drugs for 4 days. Relative cell numbers were measured by the 3-(4,5-dimethiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (15) or methylene blue (16) staining assays.

For senescence studies, primary cultures of mouse embryo fibroblasts (MEFs) were prepared from 12- to 16-day-old mouse embryos by a standard trypsin treatment procedure. Cells were propagated according to the 3T3 protocol (17) and frozen at every second passage until the culture underwent crisis. Cells frozen four to six passages before crisis were thawed, infected with different ecotropic viruses, and plated at 3 × 10^4 cells per 100-mm plate. Colonies formed by immortalized cells 2 weeks after crisis were fixed with methanol and stained with crystal violet. Some of the plates were continuously passaged for 2 months after crisis to confirm the immortalized phenotype.

cDNA PCR Analysis of *KHCS* mRNA Expression. The analysis was carried out essentially as described for the measurement of MDR1 mRNA (18). Two oligonucleotides flanking the region of human *KHCS* cDNA (19) homologous to the anti-Khcs GSE were used as PCR primers: 5′-AGTGGCTTGAAAATGAGCTC-3′ (sense) and 5′-CT-TGATCCCTTCTGGTAGATG-3′ (antisense). These primers amplified a 326-bp fragment from cDNA but not from genomic DNA of human cells, indicating that they correspond to different exons. The yield of the *KHCS*-specific PCR product was determined relative to that of β_2-microglobulin, an internal control (18). Kinetic analysis (18) indicated that the optimum number of PCR cycles for exponential amplification of cDNA derived from 25 ng of cellular RNA was 28 for *KHCS* and 23 for β_2-microglobulin. These reactions were carried out in separate tubes in triplicate. ^{32}P-labeled PCR products were mixed prior to electrophoretic analysis. Band intensity was quantitated using Betascan (Betagen, Waltham, MA).

RESULTS

Construction of a Normalized Retroviral cDNA Library and Isolation of GSEs Conferring Etoposide Resistance. Randomly initiating and terminating cDNA fragments, derived from poly(A)$^+$ RNA of mouse NIH 3T3 cells, were ligated with a synthetic adaptor containing ATG translation initiation codons in three different reading frames, and 200- to 500-bp cDNA fragments were amplified by PCR. cDNA normalization was carried out by differential reassociation followed by Southern hybridization with probes corresponding to genes expressed at different levels (5, 6). As illustrated in Fig. 1, the cDNA population that remained single-stranded after 100 h of reannealing shows similar hybridization intensities with tubulin, c-*myc*, and c-*fos* cDNA probes, corresponding to high-, medium-, and low-expressed genes, respectively. This normalized cDNA population was cloned in a retroviral expression vector LNCX (9), which contains translation termination codons in all three reading frames within 20 bp downstream of the cloning site (Fig. 1).

We have estimated the size of the normalized cDNA library needed for GSE selection from the incidence of GSEs in a random fragment library prepared from 6-kb topo II cDNA (4). The observed frequency of GSEs in the topo II library was ≈1/500, but this may be an underestimate since the recovery of topo II-derived GSEs was hindered by their

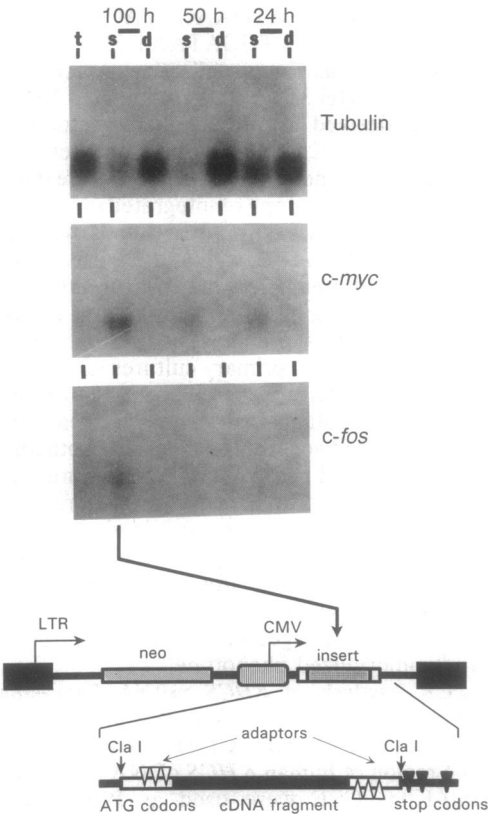

FIG. 1. Construction of normalized cDNA library. (*Upper*) Normalization of cDNA fragments from NIH 3T3 cells. Total cDNA (lane t) was reannealed for the indicated periods of time, and the single-stranded (lanes s) and double-stranded (lanes d) fractions were analyzed by Southern hybridization with the indicated probes, corresponding to high-, medium-, and low-abundance mRNA. (*Lower*) Structure of an integrated provirus containing a cDNA fragment in LNCX vector. LTR, long terminal repeat; CMV, cytomegalovirus.

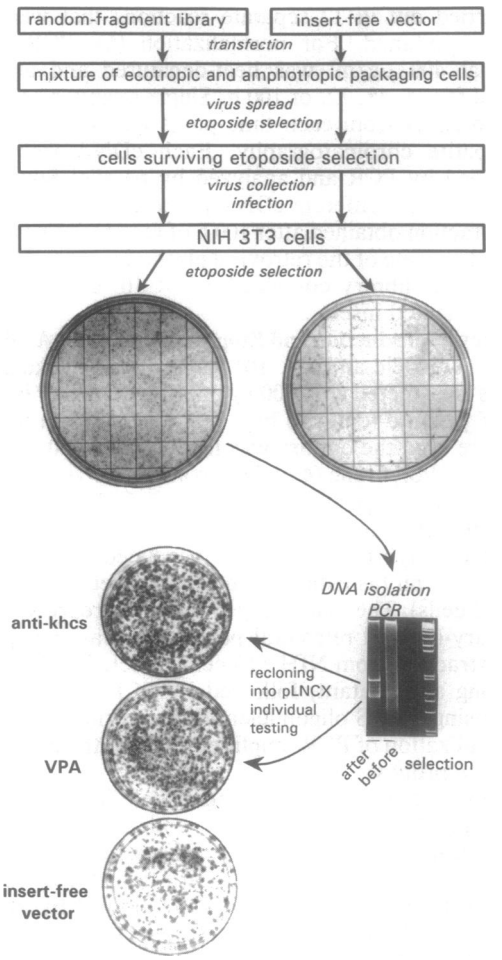

FIG. 2. Selection of GSEs inducing etoposide resistance. (*Top*) Scheme of selection. (*Middle*) Crystal violet staining of plates containing etoposide-selected NIH 3T3 cells, uninfected or infected with the library-derived virus produced by etoposide-selected packaging cells. (*Bottom right*) Ethidium bromide staining of PCR products corresponding to the proviral inserts present in library-infected NIH 3T3 cells, before and after etoposide selection. (*Bottom left*) Crystal violet staining of plates containing etoposide-selected NIH 3T3 cells, infected with LNCX-based viruses carrying the indicated PCR-amplified inserts or without an insert.

growth-inhibitory effects (4). Assuming that the complexity of a normalized cDNA population is ≈25,000 kb (10,000 expressed genes with average mRNA size of 2.5 kb), the expected incidence of GSEs derived from a gene such as topo II would be $1/500 \times 6/25{,}000 \approx 1/(2 \times 10^6)$ clones. Thus, a library containing $\approx 10^7$ clones should include GSEs for most of the expressed genes. The plasmid library generated in the present study contained $\approx 3 \times 10^7$ recombinant clones and therefore appeared suitable for GSE selection.

The procedure for GSE selection is schematized in Fig. 2. The plasmid library was transfected into a mixture of ecotropic and amphotropic virus-packaging cell lines, derivatives of NIH 3T3 (10), and the virus produced by the packaging cells was allowed to spread in this mixed population until all the cells were stably infected. Uninfected cells or cells transduced with an insert-free LNCX virus were used as a control. After infection, the packaging cells were selected with etoposide under the conditions that allowed ≈1 in 50,000 cells to grow. No difference was observed at this stage between the numbers of resistant colonies in the experiment and in the control. The virus present in the medium supernatant of the surviving cells was then used to infect NIH 3T3 cells, followed by etoposide selection. NIH 3T3 cells infected with the library-derived virus produced by etoposide-selected packaging cells now showed a major increase in the number of survivors relative to the control (Fig. 2), indicating that the preselected virus population was enriched for biologically active GSEs. PCR analysis of the integrated proviral inserts showed enrichment for specific fragments after etoposide selection (Fig. 2). The PCR-amplified fragments from etoposide-selected NIH 3T3 cells were recloned into the LNCX vector in the same position and orientation as in the original plasmid and tested for the ability to render NIH 3T3 cells resistant to etoposide. Three nonidentical clones were found to induce etoposide resistance (Fig. 2; data not shown), indicating that they contained biologically active GSEs.

Characterization of GSEs Conferring Etoposide Resistance. The sequences of the cloned GSEs were analyzed for homology to known nucleic acid and protein sequences. No significant homologies were found for either strand of the GSEs termed VPA (250 bp long) and VP9-11 (207 bp long). In contrast, the antisense strand of the third GSE (327 bp long) showed homology with several genes encoding heavy chains of kinesins, a family of cytoplasmic motor proteins involved in intracellular movement along the microtubules of eukaryotic cells (20, 21). The highest homology was found with the recently described human kinesin heavy-chain (*KHC*) gene (18), but not with a previously cloned *Khc* gene of the mouse (22). We refer to the mouse gene corresponding to the isolated GSE as *Khcs* (*s* stands for drug sensitivity), to the GSE itself as anti-Khcs, and to a kinesin molecule that would be formed by the combination of the corresponding heavy and light chains as kinesin-S.

To isolate additional *Khcs* cDNA sequences, the anti-Khcs GSE was used as a probe to screen a conventional cDNA library prepared from the RNA of mouse BALB/c 3T3 cells synchronized at $G_0 \rightarrow G_1$ transition (12). Only 2 of 400,000 clones hybridized with anti-Khcs. No positives were found among the same number of clones in a similar library prepared from unsynchronized cells. The sequences of the two isolated overlapping cDNA clones, together with the 5'-terminal segment of cDNA isolated by anchored PCR (13, 14), comprise the coding sequence for the N-terminal 880 amino acids of the mouse Khcs protein. A dot-matrix alignment of the predicted amino acid sequence of Khcs with previously cloned human (19) and mouse (22) Khc is shown in Fig. 3. Khcs is most highly homologous to the human protein (97% amino acid identity), suggesting that these kinesins are likely to be functionally equivalent. In contrast, Khcs shows approximately the same divergence from the other mouse Khc as from the Khc proteins of invertebrates, including *Drosophila* (23), squid (24), and sea urchin (25). Interestingly, the bulk of the sequence contained in the anti-Khcs GSE corresponds to the most highly diverged region among different Khc proteins (Fig. 3; data not shown).

Phenotypic Effects of the anti-Khcs GSE. To determine the spectrum of drugs to which anti-Khcs confers resistance, we repeatedly infected NIH 3T3 cells with a retrovirus carrying anti-Khcs or without an insert. The infected cell populations were then analyzed for resistance to different drugs by a 4-day growth inhibition assay. Conducting such assays on unselected mass populations rather than individual clones of infected cells allows us to overcome the potential problem of clonal variability in the interpretation of the assays, but it decreases the apparent magnitude of the GSE effect. Cells infected with the virus carrying anti-Khcs showed a moderate but reproducible increase in their resistance to the cytostatic effects of etoposide and amsacrine and, to a lesser extent, of adriamycin, camptothecin, and cisplatin (Fig. 4). All of these drugs are known to induce DNA damage, albeit by different mechanisms. Under the same assay conditions, no apparent increase in resistance was observed with colchicine or actinomycin D (Fig. 4).

The ability of anti-Khcs to induce resistance to different DNA-damaging drugs suggested that this GSE may have an effect on other types of cytostatic or cytotoxic responses. We have therefore tested the anti-Khcs GSE for the ability to interfere with *in vitro* senescence of MEFs. Senescent MEFs,

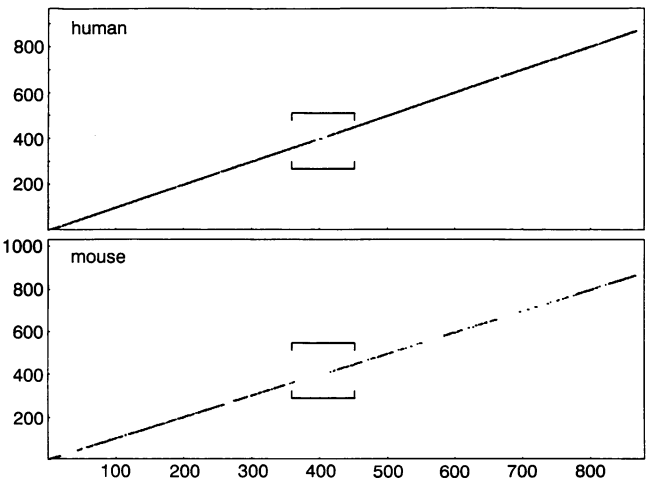

FIG. 3. Dot-matrix alignment of the predicted amino acid sequence of the mouse Khcs protein (y axes) with the previously reported human and mouse Khc proteins (x axes). The alignment was generated using the GENEPRO sequence analysis program. The region corresponding to the anti-Khcs GSE is indicated with brackets.

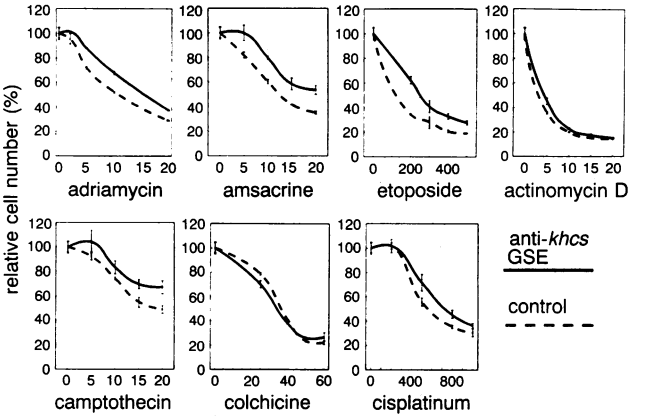

FIG. 4. Effect of different drugs on 4-day growth of NIH 3T3 cells infected with insert-free vector virus or with a virus carrying anti-Khcs. Cell growth in the absence of the drug differed by <5% for the compared populations. Drug concentrations are given in ng/ml. A representative series of parallel assays, carried out in triplicate, is shown. The experiment was repeated a minimum of three times with each of the drugs, with qualitatively similar results.

prior to undergoing crisis, were infected with insert-free or anti-Khcs-carrying retroviruses. As illustrated in Fig. 5, the cell population transduced with anti-Khcs shows a pronounced increase in the proportion of cells surviving the crisis. MEFs immortalized by anti-Khcs showed no apparent morphological features of neoplastic transformation. Furthermore, NIH 3T3 cells carrying anti-Khcs showed no detectable transformation-associated changes based on the analysis of cell morphology, serum dependence, and tumorigenicity (data not shown).

Decreased *KHCS* mRNA Expression in Etoposide-Selected HeLa Cells. Since the inhibition of *Khcs* expression with a GSE-encoding antisense RNA caused etoposide resistance, we were interested in determining whether downregulation of this gene may constitute a natural mechanism of resistance in etoposide-resistant cell lines selected by standard procedures. In the course of our studies on topo II-derived GSEs (4), we have selected several independent etoposide-resistant variants of human HeLa cells. The parental cell populations were isolated by G418 selection after transduction with LNCX vectors carrying either no insert (CX) or different GSEs derived from topo II cDNA (ε11, 6, and ε29) (4). Variants with increased resistance to etoposide were obtained from these populations by multistep drug selection to the final etoposide concentration of 200 ng/ml (in the case of CX) or 1 μg/ml (in the case of cells carrying topo II-derived GSEs).

Since the expression of *Khcs* mRNA was too low to be detected in any of the tested tissues or cell lines by Northern hybridization with a *Khcs*-specific probe (data not shown), we designed a cDNA PCR assay (18) for measuring relative levels of *KHCS* mRNA in human cells. This assay was used to test for changes in *KHCS* expression in all four pairs of

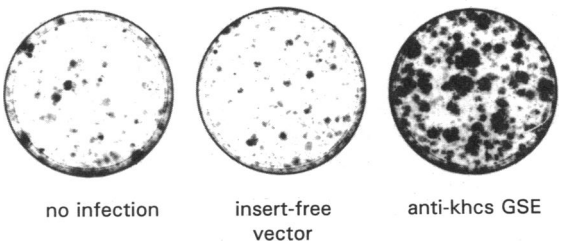

FIG. 5. Formation of immortalized cell colonies by MEFs, uninfected or infected with LNCX-based viruses carrying anti-Khcs or without an insert. One representative experiment of five is shown.

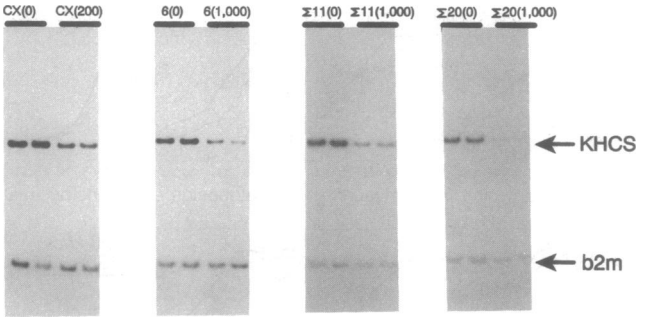

FIG. 6. PCR amplification of *KHCS* (326 bp) and β_2-microglobulin (b2m) (120 bp) cDNA-specific PCR products from the indicated etoposide-selected and unselected HeLa cell populations. One representative experiment of three is shown.

unselected and etoposide-selected HeLa cell variants (the resistant cells were removed from the drug 10 days before RNA extraction). As illustrated in Fig. 6, the yield of the *KHCS*-specific PCR product, relative to β_2-microglobulin (internal control), was decreased 2- to 3-fold in each of the four etoposide-selected populations. This result indicates that decreased *KHCS* expression is a natural mechanism for etoposide resistance in mammalian cells.

DISCUSSION

We have used GSE selection from a retroviral library of normalized cDNA fragments to identify mammalian genes whose downregulation results in drug resistance. We have isolated three GSEs, two of which are derived from presently unknown genes and one was from a member of the kinesin gene family that had not been previously associated with drug response. Although the selection was carried out with etoposide, the same agent that was previously used to isolate GSEs from topo II cDNA (4), none of the GSEs cloned in the present study was derived from topo II. This apparent paradox can be explained in part considering that topo II-derived GSEs, while conferring etoposide resistance, had a detrimental effect on cell growth (4). In contrast, no such effect was observed under standard conditions with any of the GSEs cloned in the present study, indicating that these elements should have a selective advantage over topo II-derived GSEs.

The identification of a kinesin heavy chain (KHCS) as the product of the gene inhibited by one of the cloned GSEs reveals an additional biological role for the kinesin family of proteins. Kinesins were previously shown to transport vesicles from the minus to the plus end of microtubules (20, 21). Kinesins were also proposed to play a role in intracellular movement of such diverse structures as RNA, proteins, or different organelles, as well as in the sliding of microtubules relative to each other (20, 21). We have found that *KHCS* inhibition induces resistance to the cytostatic effects of different DNA-damaging drugs and promotes immortalization of primary MEF cells. These effects are consistent with a hypothesis that kinesin is involved in a common mechanism of growth arrest induced by exposure to DNA-damaging drugs or by cellular senescence. The phenotypic changes induced by anti-Khcs resemble those associated with inactivation of a tumor suppressor p53 (26), suggesting that these proteins may be associated with a common regulatory pathway. The effects of anti-Khcs are of particular interest in light of our observation that downregulation of KHCS is a spontaneous mechanism of drug resistance in etoposide-selected mammalian cells.

The results of the present study demonstrate that GSE selection from a retroviral library of normalized cDNA fragments provides an efficient approach to expression cloning of mammalian genes. In this strategy, the same expression library and even the same transduced cell population can be used to select for GSEs inducing different phenotypic changes. Thus, we have used the NIH 3T3 cDNA library, developed in the present study, to isolate GSEs that endow NIH 3T3 cells with different properties associated with neoplastic transformation (unpublished data). The normalization of the cDNA population not only enables the cloning of cDNA sequences expressed at a very low level (as in the case of *Khcs*) but also attenuates the differences between cDNA libraries prepared from different tissues, making such libraries suitable for GSE selection in heterologous cell types. GSE selection can be used not only to elucidate previously unknown gene functions but also to identify functional domains of the corresponding proteins and to develop efficient reagents for targeted gene suppression (3, 4). The GSE approach therefore is a highly versatile tool for analysis of biological processes at many different levels.

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