Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6409–6413, July 1995
Biochemistry

Cloning and sequencing of *CATR1.3*, a human gene associated with tumorigenic conversion

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Communicated by Leo A. Paquette, Ohio State University, Columbus, OH, March 28, 1995 (received for review October 17, 1994)

ABSTRACT **The human squamous cell carcinoma cell line SCC83-01-82 (SCC) contains mutations in both the H-*ras* and *p53* genes, but it exhibits a nontumorigenic phenotype in *nude* mice. This cell line can be converted into a cell line with a tumorigenic phenotype, SCC83-01-82CA (CA), by treatment with the mutagen methyl methanesulfonate (MMS). This indicates that additional genetic events leading to expression of a cooperating tumor susceptibility gene(s) may be required for tumorigenicity. To identify the cooperating gene(s), an expression cDNA library was made from tumorigenic CA cells. The library DNA was transfected into nontumorigenic SCC cells and the transfected SCC cells were then injected into *nude* mice for the selection of a tumorigenic phenotype. Tumors developed in 3 of the 18 mice after injection. Several new cell lines were established from these transfected cell-induced tumors and designated as CATR cells. Tumor histology and karyotype analysis of these cells indicated that they were of human epithelial cell origin. All the CATR cells have the library vector sequence integrated in their genome. Cell line CATR1 expressed a single message from the integrated library representing a 1.3-kb cDNA insert that was absent from untransfected SCC cells or MMS-converted CA cells. This 1.3-kb cDNA insert was cloned by PCR amplification of reverse-transcribed CATR1 total RNA and was designated *CATR1.3*. The nucleotide sequence of *CATR1.3* encodes a peptide of 79 amino acids, has a long 3′ untranslated region, and represents an unknown gene product that was associated with the tumorigenic conversion due to the transfected expression library.**

Head and neck squamous cell carcinoma is a common tumor that accounts for 6% of all solid tumors in humans (1). Although smoking and alcohol are often considered risk factors (2, 3), the route by which the epithelial cell acquires its tumorigenic potential is not clear. At the molecular level, the activation of oncogenes and the inactivation of tumor suppressor genes are believed to play major roles in tumorigenicity (4). The most commonly altered genes in squamous carcinoma are c-*myc*, *p53*, and *ras*. Increased expression of the c-*myc* gene has been observed in squamous cell carcinomas (5). Mutations in the *p53* gene have been reported in 34% of mucosal head and neck squamous carcinomas (6). Alterations of these genes have not been identified in all squamous carcinoma cells; for example, *ras* gene mutations are present in only 15% of human tumors (7) and are very rare in head and neck squamous cell carcinomas (8). Despite an increasing number of newly discovered tumor susceptibility genes, the mutational events that result in squamous cell carcinomas are unknown.

The SCC-83-01-82 (SCC) cells are nontumorigenic in *nude* mice, yet they can be converted to a tumorigenic phenotype after treatment with the mutagen methyl methanesulfonate (MMS). This MMS-induced conversion is not associated with additional mutations in commonly investigated tumor susceptibility genes (9–11).

To identify the gene(s) involved with the conversion of human nontumorigenic SCC cells to a tumorigenic stage, we developed a human tumor-related gene isolation system which combines the concept of transfection-mediated expression cloning and expression of the malignant stage in *nude* mice. This approach to the study of conversion of nontumorigenic SCC cells eliminated the necessity to use a nonhuman tumorigenic mammalian assay system to evaluate the tumorigenicity of these converted cells. The 3T3 mouse cell transformation assay (12, 13) and the provirus tagging (for a review see ref. 14) methods depend on the presence of specific DNA sequences for the introduced DNA. These specific sequences serve as "tags" to locate the relevant transforming genes. Specifically, one of the limitations of the method used in the 3T3 system is that the human *Alu* repeat is used as a tag against a mouse genomic DNA background; therefore it is not suitable for identification of any tumor susceptibility gene that does not transform the mouse cells. In the case of the proviral tagging method, a viral sequence tag provides a method for identifying a restricted number of cell types, namely those cells that will support virus replication. Another method uses mammalian cell expression cloning or transfection-mediated expression cloning (15–17). These methods were developed to isolate genes according to their functional expression. These genes can then be identified either by cloning the vector sequence tag or by plasmid rescue (18). However, because of the limitation of the selection method (18, 19), this system, when used for oncogene isolation, has yielded only genes related to focus formation in mouse 3T3 cells. Here we report the establishment of a tumorigenic squamous carcinoma cell line (CATR1) that acquired tumorigenicity in *nude* mice after transfection of nontumorigenic SCC cells with a cDNA expression library. We propose that the gene designated *CATR1.3*§ may be involved in the tumorigenic conversion of SCC cells by the transfected expression cDNA library. The combination of transfection-mediated expression cloning and tumorigenic selection in *nude* mice can be used to identify unknown tumor susceptibility genes.

MATERIALS AND METHODS

Cell Culture. To establish cell cultures from progressively growing tumors produced in *nude* mice, a piece of tumor tissue was minced into 1 × 1 mm sections and digested with 0.5% collagenase in minimal essential medium (MEM) with 5% fetal bovine serum (FBS), at 37°C in a 4% CO_2-enriched air

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Abbreviations: SCC cells, SCC83-01-82 cells; CA cells, SCC83-01-82CA cells; MMS, methyl methanesulfonate; FBS, fetal bovine serum; RT-PCR, reverse transcription PCR.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U25433).

atmosphere for 3–5 hr in medium supplemented with 10% FBS; growth medium was changed every 2 or 3 days as described (9).

cDNA Library Construction. Total RNA was isolated from the SCC83-01-82CA (CA) cells as described (20). Poly(A)$^+$ RNA was selected by using the FastTrack mRNA isolation method (Invitrogen). Five micrograms of poly(A)$^+$ RNA was used to synthesize cDNA, using oligo(dT) primers and Superscript reverse transcriptase (GIBCO/BRL). The cDNA library was constructed by using the Librarian cDNA library construction kit (Invitrogen). Briefly, *Bst*XI cloning linkers (5′-GAATTCCACCACA/5′-GTGGAATTC) were added to both ends of oligo(dT)-primed cDNA. The cDNA with linkers was then purified by cDNA spin column (Pharmacia) and ligated to the *Bst*XI site of the eukaryotic expression vector pRC/RSV (Invitrogen). The ligation mixture was used to transform *Escherichia coli* strain DH10B. The cDNA library was plated out on Luria–Bertani (LB; ref. 21) plates containing ampicillin at 50 μg/ml. Then 1.1×10^6 colonies from primary plates were pooled in 200 ml of LB medium containing 7% (vol/vol) dimethyl sulfoxide (DMSO) and stored at −20°C as library stock.

Library Transfection. Plasmid DNA was purified from the cDNA library as follows: 500 ml of terrific broth (TB; ref. 21) medium with ampicillin was seeded with 10 ml of the cDNA library stock, the bacterial culture was incubated at 37°C overnight with shaking, and supercoiled plasmid was isolated by CsCl gradient ultracentrifugation as described (22). For each batch of transfection, eight 15-cm plates with 60% cell confluency were used. The plates were washed twice with 20 ml of phosphate-buffered saline (PBS) (21) and then 40 μl of LipofectAce (GIBCO/BRL) and 30 μg of *Sal* I-linearized plasmid in 5 ml of serum-free medium were added to each plate. After overnight incubation, 5 ml of fresh medium containing 20% FBS was added. The plates were incubated for another 24 hr. The medium was then replaced by 20 ml of fresh MEM containing 10% FBS. G418 (GIBCO/BRL) was added at 250 μg/ml for the selection of pRC/RSV-transfected cells. After 2 days, the cell cultures were split 1 to 4 in MEM with 10% FBS and G418 at 250 μg/ml, and after 2 weeks the G418-resistant colonies were pooled and the cells were grown to generate $>10^7$ cells.

Tumorigenicity Selection in *nude* mice. Transfected cells were harvested by trypsinization. The cells were washed and resuspended in MEM at $\approx 2 \times 10^7$ per ml. Three- to 4-week-old male gnotobiotic *nude* mice were splenectomized and then $\approx 10^7$ cells were injected subcutaneously into their flanks as described (9). For each batch of transfected cells, one to four sites on each mouse were injected. Tumors that were 1–2 cm in length were harvested for histological analysis and cell culture. Tumorigenic assays for the derivative cells were performed as previously described (9).

Southern Analysis. DNA isolation was carried out according to methods described by Sambrook *et al.* (21). Electrophoresis was carried out on an 0.8% agarose gel in Tris acetate/EDTA buffer. Southern blot hybridization was performed as described (23). Vector probes were prepared by using a United States Biochemical random primer labeling kit and linearized plasmid pRC/RSV according to the supplier.

Northern Analysis. Electrophoresis and Northern transfer were carried out by using methods described by Fourney *et al.* (24). Briefly, 1 μg of poly(A)$^+$ RNA was loaded per lane. The probes were labeled by random priming and probe at 1×10^6 cpm/ml was used in the hybridization protocol as described (25).

Reverse Transcription PCR (RT-PCR). RT-PCR amplification was carried out on a standard thermal cycler according to the methods described in the GeneAmp RNA PCR kit (Perkin–Elmer/Cetus). The cloning linker specific primer was 5′-GCCAGTGTGGTGGAATTC. The amplification cycle used in this procedure was 95°C for 2 min, then 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, through 35 cycles.

Cloning and Sequencing of PCR Product. RT-PCR product was cloned by using the PCRScript cloning kit (Stratagene) according to the manufacturer. Several clones were selected for sequencing for the purpose of sequence verification. Double-stranded plasmid bearing *CATR1.3* cDNA was sequenced by using USB Sequenase (United States Biochemical).

Homology Search. The DNA sequence and the derived protein sequence were searched for homology with the existing DNA and protein sequences stored in GenBank by using BLAST (26).

RESULTS

Transfection-Induced Malignant Conversion of an SCC Cell Line. SCC cells were strictly nontumorigenic in *nude* mice (never produced tumors). The nontumorigenic SCC cells were transfected with the plasmid DNA isolated from a eukaryotic expression cDNA library (1.1×10^6 primary colonies) produced from the cDNA of tumorigenic CA cells. The library cloning vector, pRC/RSV, contains enhancer-promoter sequences from the Rous sarcoma virus long terminal repeat (RSV LTR) that serve as a strong eukaryotic promoter for the transcription of the cDNA insert. The vector also contains a simian virus 40 early promoter directing a neomycin-resistance gene (aminoglycoside phosphotransferase gene) as a selection marker in eukaryotic cells. Because the CA cDNA population was cloned nondirectionally, the transcripts can be either the sense or antisense strand of the cloned cDNAs. After transfection, the SCC cells were selected by using G418 for 2 weeks until resistant colonies appeared. For each transfection, $\approx 10^4$ colonies were pooled and grown in G418-containing medium until more than 10^7 cells were generated. The cells were harvested and 10^7 cells were injected subcutaneously into each of 18 *nude* mice. Of the 18 mice injected, 3 developed progressively growing tumors (>1.2 cm in length) at the injection sites in 3–5 months (Fig. 1). The remaining mice did not develop tumors after prolonged observation, although all the injected cells had an integrated vector. The histology of the tumors was consistent with a poorly differentiated carcinoma and was the same as that of tumors originating from SCC cells converted after MMS treatment (9) (Fig. 2). Karyotype analysis indicated that the cells were of human origin. This CATR1.3 cell line is aggressively tumorigenic, as it produced progressively growing tumors in 100% of the injected *nude* mice (6/6) in just 10–20 days. A comparison of the tumorigenic

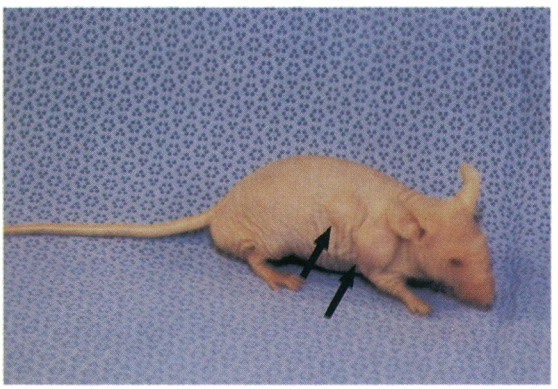

FIG. 1. One of the *nude* mice identified in Table 1. This tumor was in excess of 2.0 cm in length and represents a progressively growing tumor from the transfected cells. Two tumors can be observed near the right shoulder (indicated by arrows), where this mouse was inoculated. This tumor size is representative of the size of the tumors 3 months after the subcutaneous injection of the CA cDNA expression library-transfected SCC cells.

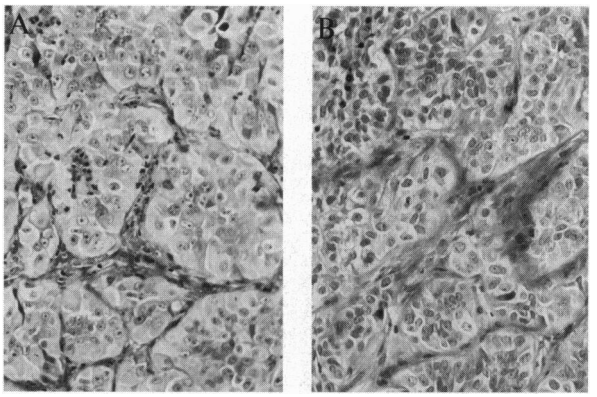

FIG. 2. Cytopathology of the progressively growing tumors (see Fig. 3) formed by the injection of 5.0×10^6 SCC cells into the subscapular area of the mouse. (*A*) Cytopathology of the tumor that is formed from SCC cells converted by MMS treatment. (×145.) (*B*) Cytopathology of the tumors created by the cDNA library-transfected SCC cells. (×145.) Both tumors showed multiple tumor nests with poorly differentiated tumor cells.

characteristics between SCC and its various derivative cells is presented in Table 1.

Integration of the Vector Sequence Tag in the Genomic DNA of Transfection-Converted Tumor Cells. The cloning vector sequence was detected by Southern analysis using vector DNA as a probe (Fig. 3). With *Eco*RI digestion, two major DNA fragments (5.5 and 1.8 kb) were detected in cell line SCC-83-01-82CATR1 (Fig. 3, lane 1). Various DNA fragments were also detected in other transfection-converted CATR tumor cells (Fig. 3, lanes 2–4). In contrast with the above observed fragment size patterns, the unintegrated library cDNA clones with the same digestion generated cDNA inserts and vector fragments of 4.2, 0.85, and 0.14 kb. These fragment size discrepancies between the expected and observed supported the idea that recombination events of the vector region occurred *in vivo*. Considering the fact that the cloning vector, pRC/RSV, cannot replicate episomally in human epithelial-derived SCC cells, we conclude that the detection and recombination of the vector sequences after extensive cell proliferation have occurred, evidence that the vector was integrated into the genomic DNA.

Expression from the Integrated Library DNA. The expression of the vector-related sequences was examined by Northern analysis of the poly(A)$^+$ RNA isolated from CATR1. A 1.5-kb message was detected by using the cloning vector as a probe after 24-hr exposure (Fig. 4). Although the cells were selected for neomycin resistance before tumorigenic selection in *nude* mice, this message was not from the neomycin-resistant gene region of the vector, because it was not detected when this region was used as a probe in the same experiment. However,

Table 1. Comparison of the malignant potentials of the various SCC derivatives

Cell type*	No. of mice with tumor/no. of mice injected	Latent period before tumor initiation
SCC	0/26	Not applicable
MMSSCC-83-01-82	5/11	3–4 months
TRSCC-83-01-82	3/18	3–5 months
CA	7/7	7–10 days
SCC-83-01-82CATR1	6/6	7–10 days

*MMSSCC-83-01-82, MMS-treated SCC cells; TRSCC-83-01-82, expression library of transfected SCC cells; CA, a cell line derived from tumors isolated from *nude* mice; SCC-83-01-82CATR1, a cell line derived from tumors of the TRSCC-83-01-82 cells in the *nude* mice. The injection of SCC cells into *nude* mice has been repeated at least three times with similar results.

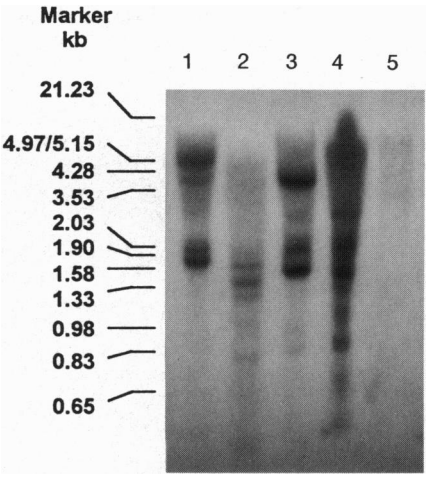

FIG. 3. Southern analysis of the DNA prepared from transfection-converted tumor cells isolated from the mouse. This figure presents evidence for the integration of the expression library vector. Ten micrograms of genomic DNA was used in each lane and the membrane was probed with labeled vector DNA. Lane 1, DNA prepared from CATR1 cells; lanes 2–4, DNA from three different cultures or subcultures of CATR cell lines; lane 5, DNA from untransfected parental SCC cells. The marker DNA was *Eco*RI- and *Hin*dIII-digested λ phage DNA.

we did detect *neo* gene expression by using RT-PCR (data not shown). Therefore, according to the expression vector construction, the 1.5-kb message represented a 1.3-kb cDNA insert with about 0.2 kb of flanking vector sequence. This assumption was further supported by the results of RT-PCR. Using the cDNA linker-specific primer, which served as both the upstream and downstream primers of the cDNA insert, RT-PCR amplified two fragments from CATR1 total RNA (Fig. 5). The larger fragment was about 1.3 kb, as predicted from the message size detected by Northern analysis. The smaller fragment was about 400 bp and the corresponding message was not detected by Northern analysis, presumably due to expression below the detectable level.

Cloning and Sequencing of the 1.3-kb cDNA Insert. The *CATR1.3* cDNA was the most highly expressed gene in the SCC cells transformed by transfection of the CA cDNA expression library. We presume that introduction of *CATR1.3* into the SCC phenotype transformed the nontumorigenic cells into a CA phenotype. These transfected cells retain their tumorigenicity when passaged *in vitro*, unlike the CA phenotype cells converted with MMS, which decrease their tumorigenic potential when similarly passaged. The *CATR1.3* DNA, a 1.3-kb RT-PCR product, was inserted into PCR-Script SK(+) (Stratagene). The cloned PCR product was designated *CATR1.3* and the DNA sequence was determined (Fig. 6). The nucleotide sequence revealed that *CATR1.3* is 1306 bp in length and encodes a peptide of 79 amino acids. The first ATG codon starts at nucleotide 46 and the cDNA exhibits a long 3′ untranslated region. The sequence AATAGA, a potential polyadenylylation signal, was found 11 nucleotides upstream from the 3′ end of the sequence. However, no poly(A) tail was present in the sequence, presumably caused by over "polishing" the cDNA ends with T4 DNA polymerase in the process of producing the cDNA expression library. An extensive homology search, using both the nucleotide and the putative peptide sequence against the GenBank data base, revealed no significant homology with any existing sequences. Therefore, *CATR1.3* represents a previously unknown gene that was expressed from the transfected tumor cDNA after the tumorigenic conversion of the SCC cells.

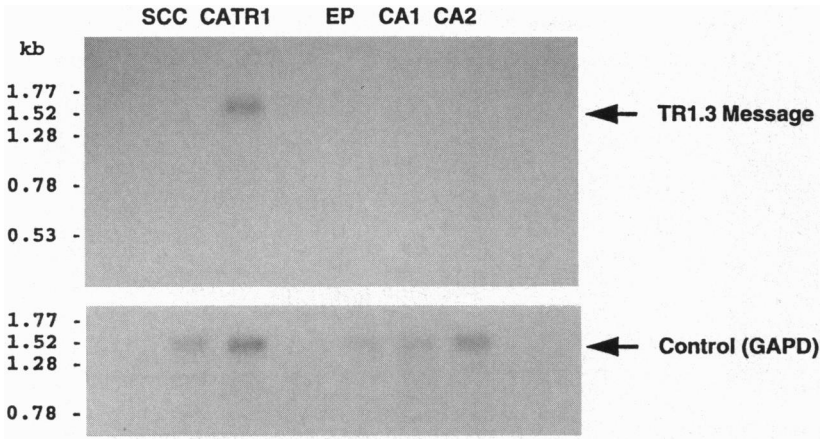

FIG. 4. Evidence by Northern analysis for the expression of a 1.5-kb message (representing a 1.3-kb cDNA insert) from the integrated library (*Upper*). The apparent larger size was caused by the flanking vector sequences. The same filter was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA as a control (*Lower*). One microgram of poly(A)$^+$ RNA was used in each lane. SCC, RNA from untransfected parental SCC cells; CATR1, RNA from CATR1 cells; EP, RNA from human epithelial cells; CA1 and CA2, RNAs from two different CA cell lines.

DISCUSSION

By the transfection of an expression cDNA library, we have converted the nontumorigenic SCC cells into the CA tumorigenic phenotype. The DNA-mediated malignant conversion may be similar to the conversion of SCC cells induced by mutagen treatment with MMS, since the DNA transfection-mediated and MMS-induced conversions have similar latent periods (3–5 months vs. 3–4 months, Table 1) and the derivative cell lines are also similar in morphology (data not shown) and tumorigenicity (compare SCC-83-01-82CATR1 with CA in Table 1).

The presence of a vector sequence tag in the derivative CATR cells indicated that the integration of the cloning vector used in the construction of the expression library had occurred. The cloning vector cannot maintain itself episomally after such extensive population doubling because the SCC cells do not support its replication.

In the transfection experiments using the same cloning vector, 15 of the 18 mice receiving transfected cells did not have tumors. Moreover, although all the injected cells should have vector sequences because of the G418 selection, only a very small number from the 10^7 injected cells are tumorigenic as indicated by the latent period of TRSCC-83-01-02 cells (Table 1) in the three positive mice. Therefore, the tumorigenicity must be caused either by the specific cDNA insert expression or by insertional activation or inactivation of a particular endogenous gene. The first possibility is consistent with both Northern analysis and RT-PCR results. *CATR1.3* was detected by using whole vector (including the *neo* region) as a probe after a relatively short exposure (overnight). The filter was then stripped and rehybridized with *neo* region-specific probe generated by PCR. This probe did not detect *neo*-related RNA after overnight exposure. When the third probe, glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) cDNA was used as the signal, it was again detected after overnight exposure. The most likely explanation is that the expression levels of both *CATR1.3* and *GAPD* were relatively high compared with that of the *neo* gene necessary for resistance to G418 at 250 μg/ml. There are other possibilities, including the loss of the *neo* region or its simian virus 40 promoter during clonal expansion in *nude* mice by recombination. This is possible because there is no selective pressure for G418 resistance during tumor formation. However, *neo* gene expression was detected by RT-PCR in all the CATR cells, supporting the first explanation. The DNA sequence and the derivative peptide sequence of *CATR1.3* have not been reported previously and there is no significant homology with existing sequences. We must point out, however, that the expression of *CATR1.3* may be specifically associated with the conversion of SCC cells and may not be a general factor related to the evolution of cancer.

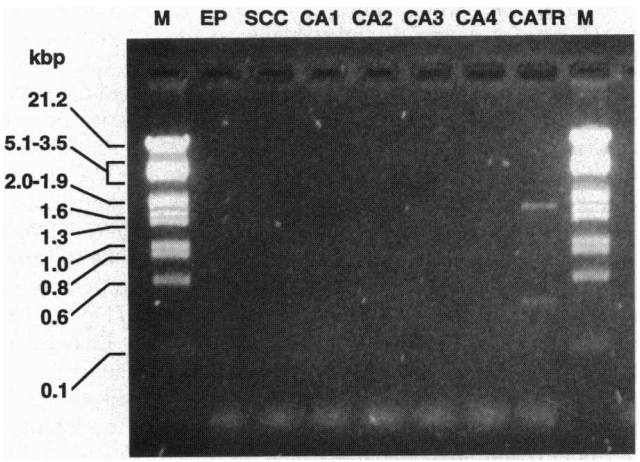

FIG. 5. PCR amplification of the 1.3-kb cDNA insert from CATR1 total RNA. A 0.5-μg sample of total RNA was used in each reverse transcription reaction with a vector downstream primer (RS2). One-fourth of the reaction mixture was then used for PCR with linker-specific primer (IA1) flanking the cDNA insert. The 1.3-kb insert was amplified only from the RNA of CATR1 cells, not from that of other untransfected control cells. M, *Eco*RI- and *Hin*dIII-digested λ DNA marker. The RNA origins are marked for each lane. EP, normal human epithelial cells; SCC, parental untransfected SCC cells; CA1–4, four different tumorigenic CA cell lines derived from tumors of MMS-converted SCC cells; CATR, cell line CATR1.

The combination of transfection-mediated expression cloning in SCC cells and tumorigenic selection in *nude* mice appears to be a useful way of isolating tumor susceptibility genes without prior knowledge of the gene family. The major differences between our expression cloning system and the 3T3-based expression cloning system developed by the group of Aaronson (18) are the transfection cell system (human cells versus mouse cells) and the selection method (tumor formation in *nude* mice versus focus formation in 3T3 cells). While the 3T3 system has been useful in identifying oncogenes related to contact inhibition of growth, such as the *ras* and *raf* families (18, 19), its ability to identify other tumor-related genes appears to be very limited. The system reported here addresses this limitation. There are obvious advantages associated with the system of expression cloning in SCC cells and *nude* mouse selection for the isolation of unknown tumor susceptibility genes. First, this approach largely solved the size limitation problem associated with genomic DNA transfection, because the sizes of most messages are within the clonable range (1–10 kb). Second, this approach does not require particular host cells or species background.

The successful conversion of the nontumorigenic SCC cells to a tumorigenic phenotype by the transfection of a cDNA expression library and the subsequent cloning of the integrated

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                                                                1 ACCAGCACACCACTGTGTAATTTCTATACGAGGTTTGGCTTGGAT 45

ATG GTG CTA AAT GAA GAG ATT CCT CGA CAT TTG CTT CTC ACT CAA AAT AAT GAC ATA ATT CCG AAG CAC CAT 117

Met Val Leu Asn Glu Glu Ile Pro Arg His Leu Leu Leu Thr Gln Asn Asn Asp Ile Ile Pro Lys His His 24

ATC TTA ATC TTA CCA GCA GTA GAC AGT TAT CAA AAA AGT GTT AAT GAT TTA AGA GCT CTA ACA TTT TCT AAG 189
Ile Leu Ile Leu Pro Ala Val Asp Ser Tyr Gln Lys Ser Val Asn Asp Leu Arg Ala Leu Thr Phe Ser Lys 48

TTT CAA GAA TTA AAG CAT GCC CAT GAA TTA AGA AAC CTT TGT GTC TCC CAA TCA AGG TTT CTA GCT ATT ATG 261

Phe Gln Glu Leu Lys His Ala His Glu Leu Arg Asn Leu Cys Val Ser Gln Ser Arg Phe Leu Ala Ile Met 72

TGG TTT GGG ACT AAC ACC AAC TGA 285
Trp Phe Gly Thr Asn Thr Asn Ter 79
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FIG. 6. Coding region nucleotide sequence and the derived peptide sequence of the 1.3-kb RT-PCR product (*CATR1.3*). Both nucleotide sequence and peptide sequence were used to search the GenBank data base, using the BLAST program, and no homology was found. The full sequence of *CATR1.3* is 1306 bp in length and encodes a peptide of 79 amino acids. The first ATG codon starts at nucleotide 46 and the cDNA has a long 3′ untranslated region (not shown). A potential polyadenylylation signal, AATAGA, was found 11 nucleotides upstream from the 3′ end. The full sequence of *CATR1.3* is available through GenBank (accession no. U25433).

and expressed cDNA insert *CATR1.3* indicate the feasibility of this method. These data further raise the possibility that yet unknown genes play a role in the conversion from the nontumorigenic stage to the tumorigenic stage. It appears as though the *CATR1.3* cDNA was the most highly expressed gene in CATR1 cells transformed from SCC cells after the transfection of the CA cDNA library. We recovered the *CATR1.3* DNA from the tumorigenic clones that retained the tumorigenic phenotype after long-term culture. This isolated DNA can be subcloned into an expression DNA vector(s) and the plasmid characterized to ensure that the protein produced is in the proper reading frame to reintroduce it into SCC cells. However, RT-PCR results indicate that there may exist several other cDNAs expressed at low levels in the other two CATR cell lines. A variety of chemicals, including MMS and 1-methyl-3-nitro-1-nitrosoguanidine, can cause the tumorigenic conversion of SCC cells with different efficiencies. This indicates the possible existence of parallel/multi- pathways that lead to tumor formation in the SCC background. Our theory is that the specific background of SCC cells (with several gene alterations) requires additional alterations in the mRNA (mutation or expression level change) of one or more of several other genes to exhibit the tumorigenic phenotype. However, we are aware that the inability of the SCC cells and other human tumor cells to form tumors in *nude* mice may merely be due to small changes in gene function that occur in an unstable genetic population of cells. These small alterations could presumably lead to cell lines from human tumors that can no longer form tumors in *nude* mice after transplantation.

Large pertubations induced by treatment of nontumorigenic cells with MMS or methylnitronitrosoguanidine are not sufficient to cause a permanent change in the cells as would be expected if small alterations in gene function were enough to restore the tumorigenic phenotype. In fact, the changes induced by MMS are operative only if the treated cells are placed into the *nude* mouse shortly after treatment.

Next, one would not expect the cells from nontumorigenic human tumors to exhibit consistent behavior since they represent plastic, unstable genetic populations. In fact, all of the nontumorigenic cells that are converted by MMS or by transfection behave remarkably similarly in their inability to form tumors before conversion, latent periods to tumor after treatment, tumor morphology, cultural characteristics *in vitro*, tumor induction frequencies, and chromosomal properties. These similar parameters between different human tumors are more representative of phenotypes of cells that had reached a particular stage in neoplastic progression and which require further genetic alteration to express a fully tumorigenic phenotype.

We thank Dr. Jucheng Chen for helpful suggestions and technical support for tumor pathology. This work was supported in part by National Cancer Institute Grant CA25907 (G.E.M. and C.S.).

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