J. Biochem. **137**, 79–85 (2005)
DOI: 10.1093/jb/mvi013

Gene Trap Screening for Cell Surface and Extracellular Matrix Molecules Produced by Chondrocytes

Shin-ichi Shirasawa[1,2], Masayuki Yoshimi[1], Hideaki Kamochi[1], Takeshi Muneta[2], James H. Kimura[3], Magnus Höök[4] and Tamayuki Shinomura[1,*]

[1]Tissue Regeneration and [2]Orthopedic Surgery, Department of Hard Tissue Engineering, Tokyo Medical and Dental University, Tokyo 113-8549; [3]Department of Surgery, John A. Burns School of Medicine, University of Hawaii at Manoa, Hawaii 96822, USA; and [4]Texas A&M Health Science Center, Institute of Biosciences and Technology, Texas 77030-3303, USA

Received September 7, 2004; accepted October 26, 2004

We have developed a simple and unique strategy for identifying cell surface and extracellular matrix molecules produced by chondrocytes. Our strategy comprises two methods, retrovirus-based signal sequence gene trapping and culturing of Swarm rat chondrosarcoma chondrocytes. After infection with a retrovirus vector and isolation of hygromycin-resistant clones, trapped genes could be easily identified by the 5′ rapid amplification of cDNA ends (5′ RACE) method. Furthermore, the treatment of isolated clones with gadolinium chloride enabled us to determine whether the trapped gene expression was dependent on the state of chondrogenic differentiation.

Key words: chondrocytes, dedifferentiation, gadolinium chloride, gene trap, signal sequence.

Abbreviations: 5′ RACE, 5′ rapid amplification of cDNA ends; βgyg, a β-galactosidase-hygromycin phosphotransferase fusion gene; LTC-RCS, a cell line established from a Swarm rat chondrosarcoma; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

Cartilage is a highly specialized tissue that contains abundant extracellular matrix. Its main constituents are type II collagen and the proteoglycan aggrecan, both of which are considered to be hallmarks of cartilage differentiation (*1*). Collagen molecules assemble into a fibrillar network that retains within it highly polyanionic proteoglycans that exert swelling pressure to inflate the network, thereby providing structural resistance to compressive forces. Therefore, proper functioning of cartilage requires the production of an appropriate cartilaginous extracellular matrix and the maintenance of its integrity by the cells of cartilage, the chondrocytes. The extracellular matrix, in turn, provides important signals for the proliferation, differentiation and homeostasis of chondrocytes.

Although extensive molecular analyses of cartilage matrix have been performed, cell surface and pericellular molecules in the tissue have not been widely investigated. These molecules must play important roles in cell–matrix interactions as well as in signaling in response to extracellular cues. Therefore, a comprehensive understanding of cell surface and secretory molecules expressed by chondrocytes is a prerequisite for understanding the physiological and pathological conditions of cartilage. To identify such molecules thoroughly in cartilage, we have developed a new strategy based on the signal sequence trapping reported by Skarnes *et al*. (*2*), and the culturing of Swarm rat chondrosarcoma chondrocytes established by King and Kimura (*3*).

*To whom correspondence should be addressed. Tel: +81-3-5803-5450, Fax: +81-3-5803-0187, E-mail: t.shinomura.trg@tmd.ac.jp

In this study, we first constructed a retrovirus-based signal sequence trap vector that functions well in rat chondrosarcoma chondrocytes. The vector contains a reporter and selectable marker genes whose expression is dependent on the acquisition of a signal sequence as described by Skarnes *et al*. (*2*). The transfection of this vector into rat chondrosarcoma chondrocytes enabled us to capture genes encoding cell surface and secretory proteins. Here we report our retrovirus-based signal sequence trap strategy and its effectiveness.

EXPERIMENTAL PROCEDURES

Cell Cultures—The cell line used in this study was established from a transplantable Swarm rat chondrosarcoma (RCS) and has been maintained in continuous monolayer tissue culture for a number of years (*3*). This long term–cultured cell line (LTC-RCS) was used as a model system for chondrocytes. The cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT, USA).

Construction of Gene Trap Vectors—The DNA fragments used for the construction of vectors, pSV-ssTMβ-gyg and pSA-TMβgyg (Fig. 2, A and B), were as follows: (i) a DNA fragment of about 1 kb containing the SV40 early promoter and the rabbit β-globin intron was cleaved from expression vector pKCRH2 (*4*) with *Pvu*II and *Sac*I; (ii) a small fragment encoding the signal peptide of preprotrypsin from pFLAG-CMV-1 (Sigma Chemical, St. Louis, MO, USA) was excised with *Sac*I and *Xba*I; (iii) a small fragment containing the splice acceptor sequence

 © 2005 The Japanese Biochemical Society.

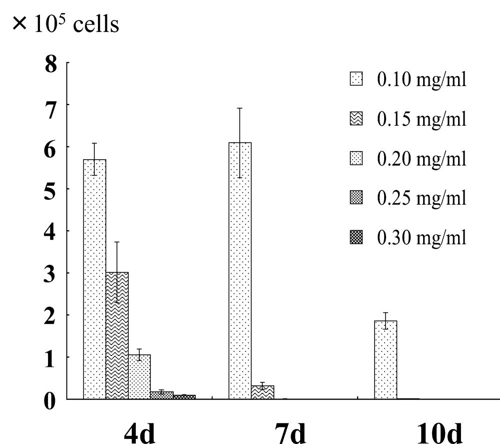

Fig. 1. **The effect of hygromycin on the survival of LTC-RCS chondrocytes.** LTC-RCS cells (5×10^5) were plated on 35-mm tissue culture dishes and then cultured in DMEM with 10% fetal calf serum and the indicated concentration of hygromycin B. The cells were fed with fresh medium every other day. On days 4, 7 and 10, the number of cells excluding trypan blue was determined after recovering of the cells from culture plates with trypsin-EDTA. Error bars indicate standard deviation of data.

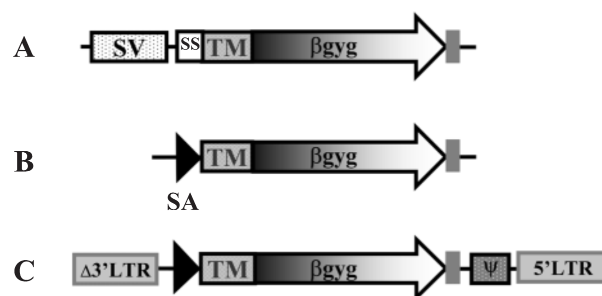

Fig. 2. **Plasmid constructs for signal sequence traps.** Plasmid vectors pSV-ssTMβgyg (A), pSA-TMβgyg (B), and prvSStrap (C) are schematically represented. SV, SV40 promoter/enhancer sequence; SS, a sequence encoding the signal peptide of preprotrypsin; TM, transmembrane domain of rat CD4; SA, splice acceptor sequence of mouse immunoglobulin; βgyg, a fusion of the β-galactosidase and hygromycin phosphotransferase genes. Retrovirus sequences are indicated by Δ3′LTR, enhancer-deleted 3′ long terminal repeat sequence, Ψ, packaging sequence, and 5′LTR, 5′ long terminal repeat sequence.

(SA) of mouse immunoglobulin was excised from pIRES1hyg (Clontech, Palo Alto, CA, USA) with *Hin*dIII and *Pst*I; (iv) the 0.7 kb DNA fragment encoding the transmembrane domain (TM) of rat CD4 was amplified from rat thymus mRNA by reverse transcriptase–polymerase chain reaction (RT-PCR) using CD4-specific primers, 5′-CATGCCATGGGAGAGTTGAGATGGAAGG-CAGAG-3′ and 5′-GCGGATCCATGAGATTGTGGCTTT-TCTGCATCC-3′; (v) an about 3 kb DNA fragment encoding the β-galactosidase gene was isolated from the plasmid pSV-β-Galactosidase (Promega, Madison, WI, USA) by partial digestion with *Cla*I and *Eco*RI; and (vi) a 1.5 kb DNA fragment containing the hygromycin phosphotransferase gene and the herpes simplex virus thymidine kinase polyadenylation signal was cleaved from expression vector pCEP4 (Invitrogen, Carlsbad, CA, USA) with *Xma*I and *Sal*I. Individual DNA fragments were first cloned into cloning vector pGEM3Zf(–) (Promega), and then several modifications were made to their 5′- and 3′-ends for the production of functional fusion genes. For instance, the 3′-end of the β-galactosidase gene and the 5′-end of the hygromycin phosphotransferase gene were modified according to the sequence reported by Natarajan and Boulter (*5*). Each DNA fragment was then excised and ligated step by step. The nucleotide sequences around the junction of each DNA fragment were confirmed by direct sequencing with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The whole sequences of the plasmid vectors pSV-ssTMβgyg and pSA-TMβgyg have been submitted to the DDBJ under accession numbers AB183487 and AB183486, respectively.

To produce a retroviral vector, prvSStrap (Fig. 2C), the *Hin*dIII fragment of pSA-TMβgyg was cloned into a derivative of the pLNCX vector (Clontech) from which the neomycin phosphotransferase gene and the cytomegalovirus promoter had been excised with *Cla*I and *Bcl*I, and the viral enhancer of the 3′ long terminal repeat (3′ LTR) was removed by partial digestion with *Xba*I and *Pvu*II. The transcriptional orientation of the fusion gene in the retroviral vector is opposite to that of the virus. The whole sequence of the vector prvSStrap has been submitted to the DDBJ under accession number AB183485.

Stable Transfections—A total of 1×10^7 LTC-RCS chondrocytes were transfected in 0.8 ml of phosphate-buffered saline (PBS) with 20 μg of pSV-ssTMβgyg (Fig. 2A), which had been linearized by digestion with *Xmn*I. Transfections were performed by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA) at 250 V and a capacitance of 950 μF. As a control, pSV-β-Galactosidase (Promega) was used to cotransfect LTC-RCS chondrocytes along with the hygromycin phosphotransferase expression vector pCEP4 (Invitrogen). After electroporation, cells were cultured in medium containing 0.2 mg/ml hygromycin B (Invitrogen), and hygromycin-resistant stable cells were isolated.

Stable transformants resulting from electroporation, as described above, or from retrovirus infection, as described below, were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Wako Pure Chemical, Osaka, Japan), following the procedure of Sanes *et al.* (*6*). Briefly, cells were rinsed twice with PBS, fixed for 15 min with 1% glutaraldehyde, and then rinsed again with PBS before staining with X-gal overnight at 37°C.

Retrovirus Production and Infection—For the production of infectious retrovirus, prvSStrap was linearized by digestion with *Xmn*I, and the resultant vector DNA was cotransfected into retrovirus packaging cell line PT67 (Clontech) with the neomycin phosphotransferase expression vector pSTneoB (*7*). After selection with G418 (Calbiochem, Darmastadt, Germany), retroviral producer cell lines were isolated. Virus titers were determined for LTC-RCS cells with hygromycin selection. As described above, the hygromycin phosphotransferase gene does not have its own promoter, therefore the virus titer reflected the efficiency of trapping of endogenous genes rather than the infection number of the retrovirus.

In general, a single insert per cell is a critical requirement for further analyses in a gene trap experiment. To

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

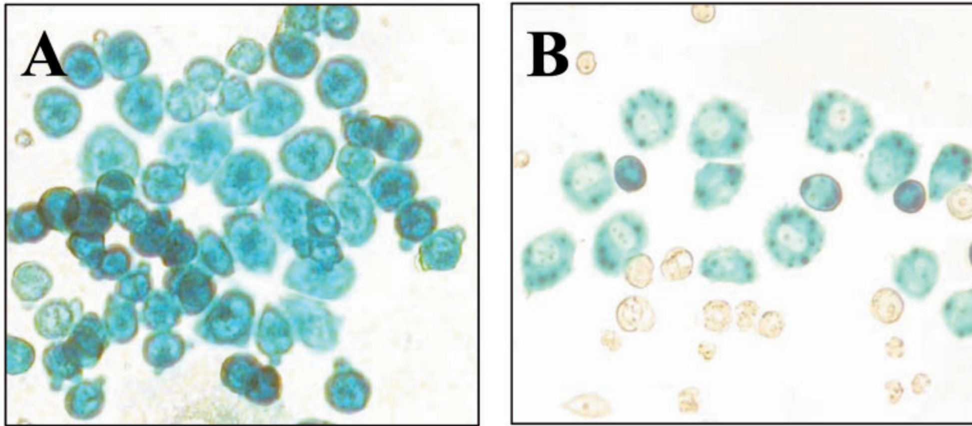

Fig. 3. **Localization of β-galactosidase activity in LTC-RCS chondrocytes.** Stable transformants with pSV-βgal (A) and pSV-ssTMβgyg (B) were cultured for 4 days, and then stained with X-gal to reveal the β-galactosidase expression patterns. Stable cell lines transfected with pSV-βgal showed a uniform distribution of β-galactosidase activity in the cytoplasm. In contrast, a signal sequence and a transmembrane domain in pSV-ssTMβgyg changed the localization of β-galactosidase activity from the cytoplasm to the endoplasmic reticulum and cytoplasmic vesicles.

achieve this, viral infection was performed at a low multiplicity of infection to restrict transfections to single integrations. However, lowering of the multiplicity decreases the efficiency of gene trapping. Thus, we carried out preliminary experiments, varying the infection conditions to obtain as many single integrations as possible, and then selected conditions under which more than 95% of the trapped genes occurred as single copies per cell.

Identification of Trapped Genes—To recover and identify trapped genes, rapid amplification of the 5′ end of the cDNA (5′ RACE) was performed using a 5′ RACE system (Invitrogen). Briefly, total RNA was isolated from each clone grown on a 100-mm tissue culture plate using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). First-strand cDNA was synthesized from the total RNA using a β-galactosidase-specific primer, lacZ (R1); 5′-CGCATCG-TAACCGTGCATCTGCCAGT-3′. After cDNA synthesis, PCR amplification of the target cDNA was accomplished using nested primer SSRT-1, 5′-TCCCAGTCACGACGT-TGTAAAACGACGG-3′, and an AAP primer (Invitrogen) first, and then CD4-specific primer CD4 (R4), 5′-TAGG-GAGAAGGTGATCCAGGACTG-3′, and an AUAP primer (Invitrogen). The PCR products were purified by agarose gel electrophoresis and then prepared for sequencing with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) using CD4 (R5), 5′-CTG-CCTTCCATCTCAACTCTCCCATGG-3′ as a primer. The reaction products were then run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Northern Hybridization—Total RNA from newborn rat rib cartilage, LTC-RCS chondrocytes, and cloned cell lines were isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. Northern blot analysis was performed as described previously (*8*). Briefly, 10 μg of total RNA was denatured, separated by electrophoresis on a 1% agarose gel, and then transferred to a Hybond N membrane with a vacuum blotter, Vacu-Gene XL (Amersham Biosciences, Piscataway, NJ, USA), as recommended by the manufacturer. The following cDNA fragments and 5′ RACE products were used as probes for hybridization; 0.5 and 0.4 kb cDNA fragments encoding the 3′ noncoding regions of rat α1(II) collagen (nucleotides 36–555; GenBank AJ224879) and rat α2(I) collagen (nucleotides 4081–4463; GenBank AF121217), respectively. After hybridization, the membranes were washed three times at a high stringency of 65°C in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate) containing 0.1% SDS, and then exposed to X-ray films at –80°C or to imaging plates (Fuji Film, Tokyo, Japan) at room temperature.

Gadolinium Chloride Treatment—LTC-RCS chondrocytes were plated at 2×10^4 cells/cm^2 in 100-mm tissue culture plates. After overnight culture in DMEM medium supplemented with 10% fetal calf serum, the cells were treated with different concentrations of gadolinium chloride as described in the text for 4 days with replacement of the medium on day 2. The effects of gadolinium chloride on the chondrogenic phenotype were examined by Northern blot analyses of type II collagen and type I collagen expression. Furthermore, the effects on cloned cell lines that have insertions in the α1(IX) collagen and α2(XI) collagen genes were monitored by X-gal staining.

RESULTS

To identify secreted and transmembrane proteins expressed by chondrocytes, we considered the application of secretory trap vectors previously reported by Skarnes *et al*. (*2*) to a long term–cultured cell line (LTC-RCS) derived from a Swarm rat chondrosarcoma (*3*). The original vectors contain a β-galactosidase-neomycin phosphotransferase fusion gene (βgeo) as a reporter gene, but LTC-RCS chondrocytes are insensitive to neomycin. More than 1.0 mg/ml of G418 is required for complete selection of neomycin-resistant cells (data not shown). Thus we have changed the reporter gene in the trap vector to a more effective one. For this purpose, we first examined the sensitivity of LTC-RCS chondrocytes to various drugs. As can be seen in Fig. 1, it is apparent that 0.2 mg/ml of hygromycin B is enough to kill LTC-RCS cells within a week.

Construction of a Retrovirus-Based Signal Sequence Trap Vector—We first constructed a plasmid vector, pSV-ssTMβgyg (Fig. 2A), that corresponded to pActSSTMβgeo reported by Skarnes *et al*. (*2*) except that our vector contained an SV40 promoter, the signal sequence of preprotrypsin, and a fusion of the β-galactosidase and hygromycin phosphotransferase (βgyg) genes, instead of the human β-actin promoter, the signal sequence of rat CD4, and the βgeo reporter gene. This plasmid vector was

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

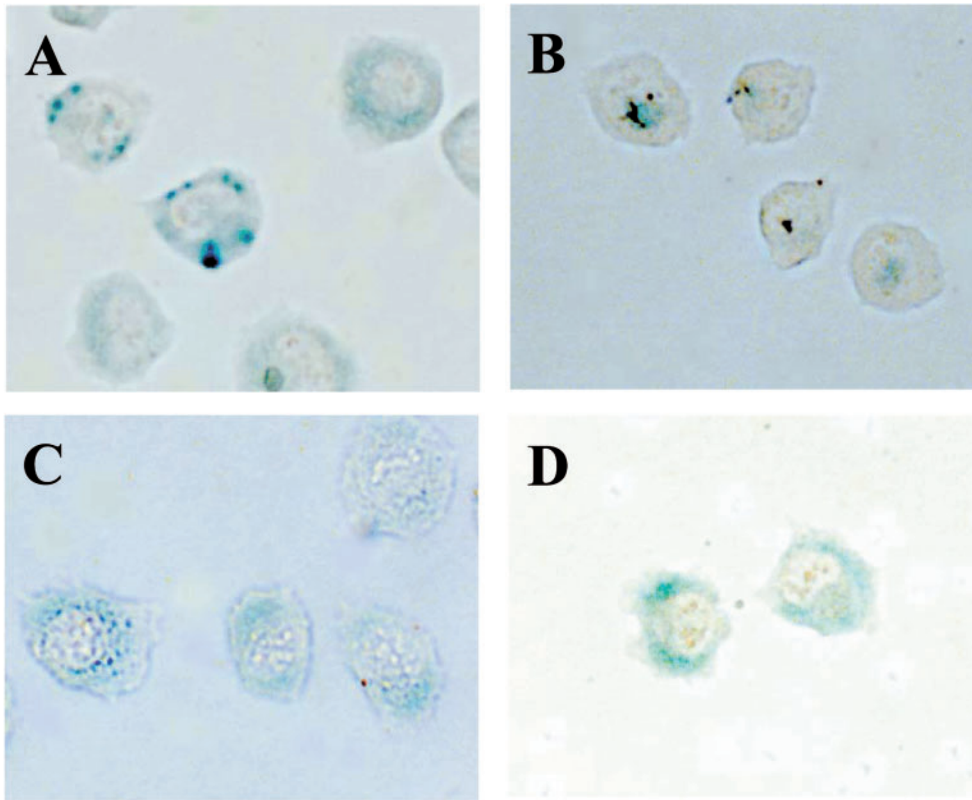

Fig. 4. **A variety of subcellular localizations of β-galactosidase activity in LTC-RCS chondrocytes stably transformed with retrovirus vector rvSStrap.** In each clone, retrovirus insertions were found in different genes encoding α1(IX) collagen (A), FK506 binding protein 9 (B), α2(XI) collagen (C), and a transforming growth factor-beta–responsive gene product (D).

introduced into LTC-RCS chondrocytes by electroporation. After selection with hygromycin, stable transformants were stained with X-gal (Fig. 3B). The results were consistent with the previous data of Skarnes *et al*. (*2*) in that the reporter gene, β-galactosidase, activity was detected in the endoplasmic reticulum and in cytoplasmic vesicles. Furthermore, the data indicated that each DNA fragment used for the vector construction functioned as expected. Each construct was then modified to create a new signal sequence trap vector by replacing the SV40 promoter and signal sequence in pSV-ssTMβgyg with the splice acceptor sequence of mouse immunoglobulin to give pSA-TMβgyg (Fig. 2B).

As described by Skarnes (*9*), signal sequence gene traps are basically dependent on the single insertion of a reporter gene into an intron of an actively expressing gene that encodes a signal sequence–possessing molecule. Therefore, for successful gene trapping, multiple insertion of vector DNA must be prevented. A single insert per cell was easily achieved by means of retrovirus infection at low multiplicity. Therefore, we further modified pSA-TMβgyg to create a retrovirus-based vector, prvSStrap, as shown in Fig. 2C. Briefly, the SA-TMβgyg sequence was placed in the opposite direction to viral transcription in the modified pLNCX retroviral vector that lacked the viral enhancer sequences in the 3′ LTR as described by Friedrich and Soriano (*10*). The resultant vector was then electroporated into PT67 retrovirus packaging cells with a neomycin phosphotransferase expression vector, pSTneoB (*7*). After selection with G418, virus-producing cells were cloned and the produced retrovirus was harvested from the culture medium.

Trapping of Genes Encoding Signal Sequences—To examine the ability of retrovirus vector rvSStrap to capture signal sequences, LTC-RCS chondrocytes were infected with the rvSStrap virus. After selection with hygromycin, X-gal staining revealed the presence of β-galactosidase positive colonies that exhibited a similar β-

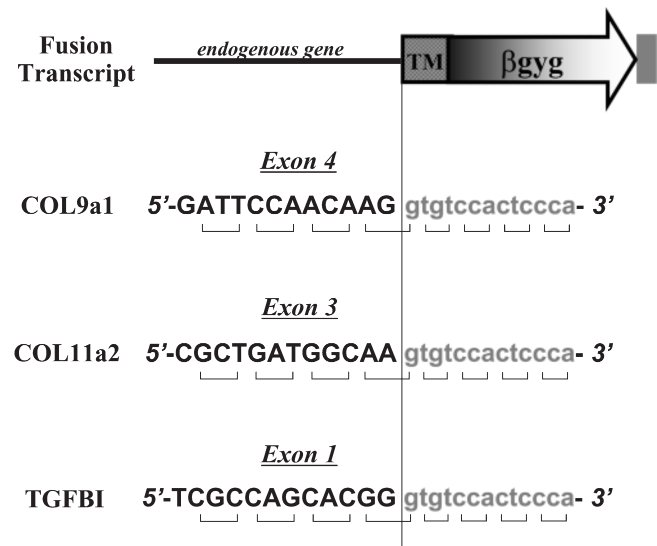

Fig. 5. **Schematic representation of fusion transcripts produced by clones shown in Fig. 4A (COL9a1), 4C (COL11a2), and 4D (TGFBI).** The nucleotide sequences at the junction of the endogenous gene (capital letters) and vector DNA (small letters), and the reading frame of each transcript are indicated.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

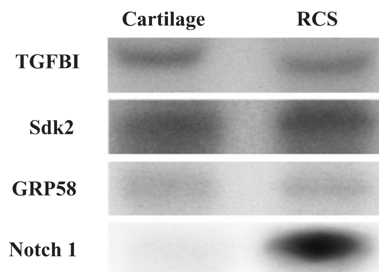

Fig. 6. **Northern blot analyses of trapped genes in cartilage tissues.** The same amount of total RNA (10 μg) prepared from fetal rat rib cartilage (Cartilage) and LTC-RCS chondrocytes (RCS) was subjected to Northern blot analyses. Hybridizations were performed using 5′ RACE products of each trapped gene as probes, TGFBI, a transforming growth factor-beta-responsive gene (*14*); Sdk2, Sidekick-2 (*17*); GRP58, glucose-regulated protein-58 (*18*); Notch1 (*19*).

Table 1. **Genes identified by signal sequence trapping.**

Trapping frequency	GenBank accession No.	Gene name
6	XM_347112	COL9a1
4	XM_216153	FKBP9
2	XM_215345	COL11a2
1	NM_009369	TGFBI
1	XM_340930	Sdk2
1	NM_017319	GRP58
1	XM_342392	Notch1
1	AB037248	ATP6k
1	AK076222	hypothetical
1	–	novel

galactosidase staining pattern to that observed with the pSV-ssTMβgyg vector. To confirm that signal sequence trap events occurred properly in these colonies, hygromycin-resistant and β-galactosidase positive colonies were randomly cloned and analyzed further. Although the X-gal staining patterns subtly differed from each other, all isolated clones showed β-galactosidase activity that was localized in the endoplasmic reticulum and in cytoplasmic vesicles, as shown in Fig. 4.

We then analyzed partial sequences of the trapped genes that were obtained by rapid amplification of cDNA 5′ ends (5′ RACE), and compared the resulting sequences with sequences in DNA databases using BLAST (NCBI). This procedure, for example, was used to identify the genes trapped by the construct for each clone shown in Fig. 4. They were α1(IX) collagen (COL9a1) (*11*), FK506 binding protein 9 (FKBP9) (*12*), α2(XI) collagen (COL11a2) (*13*), and a transforming growth factor-beta–responsive gene (TGFBI) (*14*), Fig. 4, A to D, respectively. To verify that the splice acceptor functioned properly in the isolated clones, the sequences obtained on 5′ RACE analysis were carefully compared with the exon-intron junctions of each endogenous gene. As shown in Fig. 5, insertion of the vector DNA occurred in the 1st, 3rd, and 4th introns of TGFBI, COL11a2, and COL9a1, respectively. Other clones were also shown to have used the splice acceptor sequence appropriately to give a single open reading frame with βgyg.

Effectiveness of the Signal Sequence Trap to Capture Molecules Expressed in Cartilage—Approximately 30% of hygromycin-resistant colonies were positive for β-galactosidase activity and showed various X-gal staining patterns. Both the efficiency of β-galactosidase expression and the staining patterns are consistent with those reported by Skarnes (*9*). Since Skarnes suggested that β-galactosidase negative hygromycin–resistant colonies have a type II orientation of the βgeo fusion protein as a result of the absence of a signal sequence, we did not analyze β-galactosidase negative colonies any further.

We have cloned 41 independent colonies showing β-galactosidase activity, and all of them were subjected to 5′ RACE analyses. Of these 41 clones, 13 failed to amplify at all. Of the remaining 28 clones, direct sequencing of 5′ RACE products revealed that 19 efficiently utilized the splice acceptor sequence of the vector, while the other 9 showed abnormal sequences coding the retrovirus vector itself. Of the 19 that utilized the splice acceptor sequence, BLAST searches revealed that 17 contained known genes with a signal sequence, while the remaining 2 exhibited no significant matches with any known gene (listed in Table 1). The clones that represent true signal sequence trap events have been analyzed further. In all these clones, insertions of the COL9a1, FKBP9, and COL11a2 genes were recovered 6, 4, and 2 times, respectively. The multiple independent insertions in the same gene strongly suggest that retrovirus insertions into the genome do not occur randomly.

We have successfully obtained a substantial number of signal sequence-possessing molecules that are expressed in rat chondrosarcoma chondrocytes. However, our ultimate goal is to identify molecules expressed in normal cartilage. Therefore, we have examined the expression of trapped genes in normal cartilage by means of Northern blot analysis (Fig. 6). The results indicated that most, if not all, of the trapped genes were clearly expressed in both LTC-RCS chondrocytes and normal cartilage.

Differentiation-Dependent Gene Expression of Captured Molecules—LTC-RCS chondrocytes exhibit various biochemical characteristics of normal chondrocytes. Therefore, we examined whether the gene expression of captured molecules changes in response to alteration of the phenotype of LTC-RCS chondrocytes. Previous reports indicated that 12-*O*-tetradecanoylphorbol-13-acetate and all-*trans*-retinoic acid induced dramatic morphological and biochemical changes of normal chondrocytes (*15*, *16*). These changes were accompanied by loss of the chondrogenic phenotype. However, they have little effect on LTC-RCS chondrocytes (data not shown). Recently, we found that gadolinium chloride has potent effects on LTC-RCS chondrocytes, resulting in a switch from the synthesis of type II collagen to that of type I collagen (Fig. 7A). These data suggested that the chondrogenic phenotype of LTC-RCS chondrocytes was specifically inhibited by gadolinium chloride. This was further supported on the gadolinium chloride treatment of cloned cell lines that have insertions in the COL9a1 and COL11a2 genes. In these clones, the expression of β-galactosidase was suppressed by gadolinium chloride in marked contrast to the constitutive expression of β-galactosidase in LTC-RCS chondrocytes transformed with pSV-ssTMβgyg (Fig. 7B). Type IX and type XI collagens are both well-known cartilage specific molecules (*11*, *13*). Therefore, all these data strongly

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

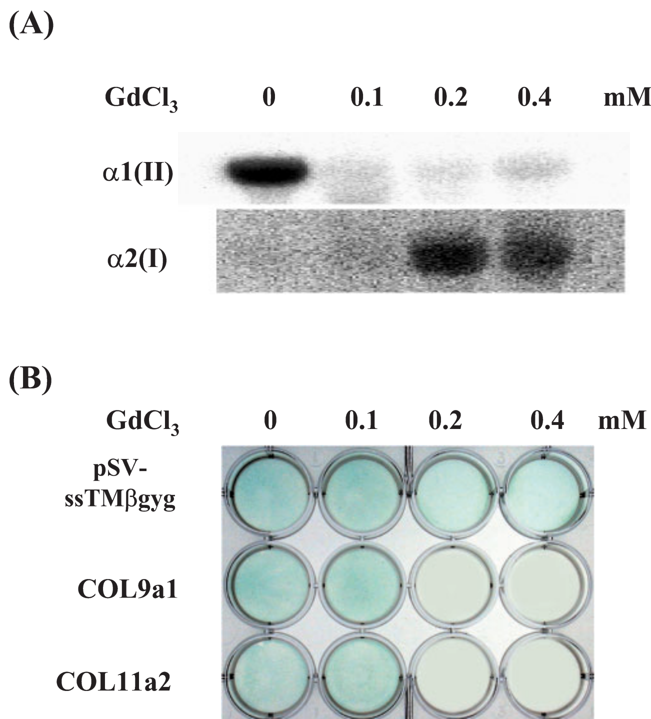

Fig. 7. **Effects of $GdCl_3$ on RCS-LTC chondrocytes.** (A) RCS-LTC chondrocytes were grown in the presence of different concentrations (0, 0.1, 0.2, and 0.4 mM) of gadolinium chloride for 4 days. Total RNA from each culture was blotted and hybridized with type II collagen, COL2a1, and type I collagen, COL1a2. (B) Isolated clones having retrovirus insertions in the COL9a1 and COL11a2 genes and, as a control, cells transformed with pSV-ssTMβgyg were grown in the presence of different concentrations (0, 0.1, 0.2, and 0.4 mM) of gadolinium chloride. On day 4, cells were stained with X-gal.

suggested that cartilage characteristic genes would be responsive to gadolinium chloride treatment and that this treatment would be useful for the enrichment of cartilage characteristic genes in our screening system.

DISCUSSION

We have developed a unique experimental system for isolating secreted and cell surface molecules expressed by chondrocytes. The system is composed of signal sequence gene trapping and culturing of LTC-RCS chondrocytes. The former was basically developed by Skarnes *et al*. (*2*), and the latter was established by King and Kimura (*3*). The signal sequence gene trapping is based on the single insertion of a reporter gene into an active transcription site. The expression of a reporter gene in this case is totally dependent on the proper use of the splice acceptor sequence and the reading frame of the coding sequence inserted in it. Therefore, we used a common splice acceptor sequence and have constructed with it three separate retrovirus-based vectors, *i.e.* rvSStrap with different reading frames. In this study, we used just one of these rvSStrap constructs. As a result, we could not capture some cartilage-specific genes such as the type II collagen and aggrecan genes, whose reading frames do not fit the construct. However, the other two constructs will make it possible for us to capture genes having different reading frames.

Sequence analysis of 5′ RACE products showed the proper use of the splice acceptor and an in-frame fusion with the reporter gene. Furthermore, all the insertions listed in Table 1 acquired a signal sequence from the endogenous genes encoding (i) extracellular matrix molecules: type IX collagen, type XI collagen, and a transforming growth factor-beta–responsive gene product (TGFBI); (ii) transmembrane proteins: a synaptic adhesion molecule (Sdk2), and a cell surface receptor (Notch 1); and (iii) subcellular components: a FK506-binding protein (FKBP9), glucose-regulated protein 58 (GRP58), and a subunit of vacuolar type proton-ATPase (ATP6K). The remaining two genes, hypothetical and novel, were subjected to a prediction program for signal peptides, SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), and were confirmed to have signal peptide sequences by this program.

Several reports have suggested that most of the genome is accessible to retroviral integration even though virus integrations occur preferentially close to hypersensitive sites (*20*). In LTC-RCS chondrocytes, at least type IX collagen, FKBP9 and type XI collagen loci appear to be hypersensitive sites, and integrations at these sites account for over 60% of the total identified clones. Therefore, even if the remainder represents random integration, our gene trap screening is not necessarily efficient for accessing most of the genome. To overcome this problem, we tested electroporation for the transfection of a vector, pSA-TMβgyg, into LTC-RCS chondrocytes as an alternative approach. However, we found that, under the conditions for electroporation that we tested, it was very difficult to prevent multiple insertions or concatemeric insertions of the vector DNA, even if inert carrier DNA was added. Too much carrier DNA resulted in lowering of the gene trap efficiency. As a result, we concluded that gene transfer through electroporation was not practical for our system. Future work will be required to develop new vectors and a delivery system that will increase the chance of unique gene trapping.

In this study, we used a cell line (LTC-RCS) derived from a Swarm rat chondrosarcoma instead of normal chondrocytes. LTC-RCS chondrocytes express various molecules common to cartilage tissue and their chondrogenic phenotype is very stable in monolayer culture in spite of the fact that normal chondrocytes easily dedifferentiate into fibroblastic cells (*21*). These features of LTC-RCS chondrocytes are very useful for isolating hygromycin-resistant clones in the course of gene trap experiments. Furthermore, we could modify the differentiated phenotype of LTC-RCS chondrocytes by treatment with gadolinium chloride. Gadolinium chloride induced a switch in gene expression from type II collagen to type I collagen in LTC-RCS chondrocytes associated with dramatic morphological changes. This was further supported by the observation that gadolinium chloride treatment suppressed the expression of β-galactosidase in cloned cell lines having insertions in cartilage-specific genes such as COL9a1 and COL11a2, but not in other genes such as FKBP9, Notch 1, and so on (data not shown). Consequently, we can easily determine whether the

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012

expression of trapped genes is responsible for the differentiation state of LTC-RCS chondrocytes. In other words, we will be able to enrich cartilage characteristic genes by excluding clones non-responsive to gadolinium chloride. Although we have confirmed that a low concentration of gadolinium chloride is not cytotoxic and its effect is reversible, the action of gadolinium chloride on LTC-RCS chondrocytes is totally unknown. Therefore, further work will be required to clarify its mechanism of action.

To identify trapped genes in the flanking region, the 5′ RACE method was used. The obtained cDNA sequences were useful for characterizing the endogenous genes. A search of the GenBank database showed that about 10% of the trapped genes found in this experiment did not match any known sequences. Such genes including hypothetical ones would represent newly discovered genes. Therefore, we are carefully analyzing their full sequences and their expression patterns in cartilage.

Although the expression of all trapped genes was not necessarily detected in cartilage on Northern analyses, we believe that our gene trap approach will be a powerful means of identifying and cataloging signal sequence–possessing molecules expressed in chondrocytes. In particular, the combination of signal sequence trapping with gadolinium chloride treatment would give us chance to identify cartilage specific molecules. Furthermore, in a more general way, by applying our trapping method to cells that can take on two different states, we will be able to identify the specific molecules expressed in the two different states. Future work will also involve the use of the gene trap approach to identify genes that are responsive to drugs, hormones, virus infection, and so on.

This work was supported in part by Grants-in-Aid for Scientific Research (13470305 and 14657360) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Muir, H. (1995) The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *Bioessays* **17**, 1039–1048
2. Skarnes, W.C., Moss, J.E., Hurtley, S.M., and Beddington, R.S.P. (1995) Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl Acad. Sci. USA* **92**, 6592–6596
3. King, K.B. and Kimura, J.H. (2003) The establishment and characterization of an immortal cell line with a stable chondrocytic phenotype. *J. Cell. Biochem.* **89**, 992–1004
4. Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takahashi, T., Kuno, M., and Numa, S. (1984) Expression of functional acetylcholine receptor from cloned cDNAs. *Nature* **307**, 604–608
5. Natarajan, D. and Boulter, C.A. (1995) A lacZ-hygromycin fusion gene and its use in a gene trap vector for marking embryonic stem cells. *Nucleic Acids Res.* **23**, 4003–4004
6. Sanes, J.R., Rubenstein, J.L., and Nicolas, J.F. (1986) Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133–3142
7. Katoh, K., Takahashi, Y., Hayashi, S., and Kondoh, H. (1987) Improved mammalian vectors for high expression of G418 resistance. *Cell Struc. Func.* **12**, 575–580
8. Shinomura, T., Nishida, Y., Ito, K., and Kimata, K. (1993) cDNA cloning of PG-M, a large chondroitin sulfate proteoglycan expressed during chondrogenesis in chick limb buds. *J. Biol. Chem.* **268**, 14461–14469
9. Skarnes, W.C. (2000) Gene trapping methods for the identification and functional analysis of cell surface proteins in mice. *Methods Enzymol.* **328**, 592–615
10. Friedrich, G. and Soriano, P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513–1523
11. Abe, N., Yoshioka, H., Inoue, H., and Ninomiya, Y. (1994) The complete primary structure of the long form of mouse alpha 1(IX) collagen chain and its expression during limb development. *Biochim. Biophys. Acta* **1204**, 61–67
12. Jo, D., Lyu, M.S., Cho, E.G., Park, D., Kozak, C.A., and Kim, M.G. (2001) Identification and genetic mapping of the mouse Fkbp9 gene encoding a new member of FK506-binding protein family. *Mol. Cells* **12**, 272–275
13. Tsumaki, N. and Kimura, T. (1995) Differential expression of an acidic domain in the amino-terminal propeptide of mouse pro-alpha 2(XI) collagen by complex alternative splicing. *J. Biol. Chem.* **270**, 2372–2378
14. Skonier, J., Neubauer, M., Madisen, L., Bennett, K., Plowman, G.D., Purchio, A.F. (1992) cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. *DNA Cell Biol.* **7**, 511–522
15. Finer, M.H., Gerstenfeld, L.C., Young, D., Doty, P., and Boedtker, H. (1985) Collagen expression in embryonic chicken chondrocytes treated with phorbol myristate acetate. *Mol. Cell. Biol.* **5**, 1415–1424
16. Benya, P.D. and Padilla, S.R. (1986) Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen: the modulated phenotype differs from that produced by subculture. *Dev. Biol.* **118**, 296–305
17. Yamagata, M., Weiner, J.A., and Sanes, J.R. (2002) Sidekicks: synaptic adhesion molecules that promote lamina-specific connectivity in the retina. *Cell* **110**, 649–660
18. Murthy, M.S. and Pande, S.V. (1994) A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme. *Biochem. J.* **304**, 31–34
19. del Amo, F.F., Gendron-Maguire, M., Swiatek, P.J., Jenkins, N.A., Copeland, N.G., and Gridley, T. (1993) Cloning, analysis, and chromosomal localization of Notch-1, a mouse homolog of Drosophila Notch. *Genomics* **15**, 259–264
20. Chang, W., Hubbard, S.C., Friedel, C., and Ruley, H.E. (1993) Enrichment of insertional mutants following retrovirus gene trap selection. *Virology* **193**, 737–747
21. Stewart, M.C., Saunders, K.M., Burton-Wurster, N., and Macleod, J.N. (2000) Phenotypic stability of articular chondrocytes in vitro: The effects of culture models, bone morphogenetic protein 2, and serum supplementation. *J. Bone Miner. Res.* **15**, 166–174

Downloaded from http://jb.oxfordjournals.org/ at Peking University on September 29, 2012