Snail genes encode zinc finger transcription factors required for the development of vertebrate and invertebrate embryos. They trigger epithelial to mesenchymal transitions (EMTs), thereby allowing epithelial cells to emigrate from their place of origin and form tissues such as the mesoderm and the neural crest. Snail genes are also involved in the EMTs responsible for the acquisition of invasiveness during tumor progression. This aspect of their activity is associated with their ability to directly repress E-cadherin transcription. Here we describe the existence of an active human Snail retrogene, inserted within an intron of a novel evolutionarily conserved gene and expressed in different human tissues and cell lines. Functional analyses in cell culture show that this retrogene maintains the potential to induce EMTs, conferring migratory and invasive properties to epithelial cells. In light of this data, we have renamed it SNAIL-like, a new player that must be considered in both physiological and pathological studies of SNAIL function in humans.

Snail genes are zinc finger transcription factors with important functions in vertebrates and invertebrates. After the identification of Snail in Drosophila (1), multiple family members have been isolated, leading to their classification in two main families, Snail and Scratch (2). A common feature of the superfamily is a role in neural development, whereas a conserved function in mesoderm formation is associated with the Snail family (2, 3). Additional roles in cell division, cell survival, left-right asymmetry, and wing and limb development have also been described in different species (3, 4).

Within the Snail family, a duplication event led to the formation of Snail and Slug in vertebrates, which evolved by dividing their ancestral functions and acquiring new ones. An example of new function can be seen during the development of the neural crest, a population of cells involved in the formation of the neural crest (5, 6). These genes are required for the acquisition of migratory properties by both the neural crest and the mesoderm, principally by triggering the epithelial-mesenchymal transition (EMT) that confers upon epithelial cells the capacity to migrate through the extracellular matrix (7, 8). Indeed, the lethality of Snail mutant mice at gastrulation is due to a defect in EMT during mesoderm formation (9). In this respect, Snail has been shown to act as a direct repressor of E-cadherin transcription both during embryonic development and tumor progression. A direct correlation has been observed between Snail activation and the acquisition of invasive and metastatic properties in human tumor cell lines of different epithelial origin (10–12). In addition, Snail is expressed at the invasive front of mouse skin tumors and human breast carcinomas (10, 13, 14).

In addition to SNAIL (HUGO Genome Nomenclature Committee approved symbol: SNAI1), an extremely related sequence was found in the human genome and classified as a nonfunctional retro-transcribed pseudogene (SNAI1P; Refs. 15 and 16). Retrogenes result from the reverse transcription of an mRNA and subsequent insertion into the genome mediated by different transposable sequences such as retrovirus, LINE, or Alu elements (17). In general, pseudogenes are not expressed due to the absence of promoter elements in the region of insertion. Even when they have the ability of being expressed after integration, the lack of regulated expression or the absence of selective pressure leads to their rapid inactivation, often as a result of accumulated changes that render the protein nonfunctional (18–24). However, an expressed retrotransposed gene can acquire adaptive mutations that lead to its functional differentiation and the acquisition of new properties (25).

Here we show that the SNAIL retrogene inserted in the human genome constitutes a transcription unit that we propose to call SNAIL-like (SNAI1L). It has been subjected to positive selection leading to the maintenance of the complete open reading frame and the conservation of its zinc finger DNA binding (3) and SNAG transactivation domains (26). It is integrated into a new gene (2q34-X) and possesses a regulated pattern of expression different from that of both SNAIL and 2q34-X. We also show that in a similar manner to SNAIL, it is able to induce EMT in epithelial cell lines and maintains the capacity of repressing E-cadherin expression. The biological potential of SNAIL-like makes it an important subject for functional studies in tumor progression. In addition, its similarity to SNAIL might have been the cause of incorrect evaluation of SNAIL expression in some studies carried out in human cell lines and tumors.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis—**Sequence comparisons were performed using BLAST (Ref. 27; www.ncbi.nlm.nih.gov/BLAST). Gene predictions were...
obtained with GENSCAN (Ref. 28; genes.mit.edu/GENSCAN.html) and those of the pufferfish and ascidian exons using sequences from the Fugu (fugu.hgmp.mrc.ac.uk) and the Ciona intestinalis (jgi doe.gov/ programs/ciona.htm) genome projects. Transcripts, genes, and ESTs present in the region analyzed (2q54) were confirmed via the Ensemble Human Genome Server (www.ensemble.org; chromosome 2, nucleotides 219550835–220050835 view). Sequence alignments were carried out using Clustal (29) and corrected by visual inspection.

**Northern Blot and RT-PCR Analyses—Poly(A)+ RNA from cell lines was purified by oligo(dT)-cellulose chromatography (30). For Northern blot assays the entire SNAI-like or SNAI coding fragments were labeled using the Rediprime II kit (Amersham Biosciences), and GAPD (glyceraldehyde-3-phosphate dehydrogenase) was used as a control of mRNA quantity. Adult human cDNAs were obtained from BD Biosciences, whereas poly(A)+ was isolated from the different tumor cell lines or stable transfecant clones (Microfast Track isolation kit, Invitrogen) and treated with DNase I before cDNA synthesis. PCR for E-cadherin, Box A, Box B, SNAI-like, and 5′-SNAI-like were performed over 35 cycles at an annealing temperature of 65–70 °C. For all SNAI amplifications from adult tissues, nested PCR reactions were carried out under the same conditions. GAPD was amplified after 30 cycles at an annealing temperature of 60 °C. Primer sequences are available upon request. Semi-quantitative analysis was carried out by densitometry of the products over different number of cycles in the linear phase of amplification.

**Generation and Characterization of SNAI and SNAI-like Stable Transfectants—SNAI and SNAI-like coding sequences were amplified by RT-PCR from poly(A)+ RNA from the A375P cell line. Primer sequences are available upon request. The amplified fragments were cloned in the pZeoS/V2+ vector (Invitrogen) and transfected with LipofectAMINE Plus (31). Stable transfectants were generated in MDCK cells after selection with Zeocine. Six independent clones were analyzed from each pZeo-SNAI and pZeo-SNAI-like and from mock pZeo transfections. The expression of E-cadherin, SNAI, and SNAI-like was analyzed in stable transfected MDCK cells by RT-PCR. For immunofluorescence analysis, cells were grown on coverslips in 6-cm cell culture dishes and fixed 24–48 h after transfection (32, 33).

**Generation and Characterization of SNAI and SNAI-like-inducible Transient Transfectants—The same amplified fragments were fused to a mutated version of the ligand binding domain of the human estrogen receptor that recognizes the synthetic ligand 4-OH-tamoxifen. The fragment corresponding to the binding site was obtained from the vector pCRE-ER(34), kindly provided by P. Chambon. The final fragments either containing SNAI or SNAI-like and another one containing the luciferase reporter gene together with either 50 ng of pZeoc-SNAI, pZeoc-SNAI-like, or control pZeoc plasmids. Luciferase and Renilla activities were assayed using the dual-luciferase reporter system kit (Promega), and the activity normalized to that of the promoter cotransfected with the control pZeo vector. The mouse SnaI expression construct was as described (10).

**RESULTS**

**Analysis and Expression of an Intronless SNAI-like Retrotransposed Sequence—**An exhaustive analysis of different ESTs and genomic databases using specific and diagnostic sequences to the SnaI genes revealed multiple new members from *Drosophila, Caenorhabditis elegans*, zebrafish, and human (2). Among these, we found a genomic sequence from human chromosome 2 (GenBank™ accession number AC006385) containing a 1863-bp segment highly related to an intronless transcript of the SNAI gene (SNAJ1). The nucleotide similarity was 81.9% in the coding region, 75% over a 254-bp stretch of the 5′-UTR, and 74.6% over 848 bp of the 3′-UTR. The region similar to SNAI was followed by an Alu-like sequence in the 5′ end and flanked by 15-bp inverted repeats at both 5′ and 3′ ends. Outside of this region, similarity drops to non-significant levels. This is strong evidence that this gene resulted from a retrotranscription of a SNAI mRNA followed by its insertion in the genome. Indeed, other groups recently described it as a processed non-transcribed pseudogene and named it SNAIIP (15, 16).

Inspection of the coding region of SNAIIP showed that the changes from the parental SNAI sequence involved 11.8% of the residues not including two insertions of 3 and 9 bp and one deletion of 30 bp. None of these changes disrupted the open reading frame. Evidences shown below indicate that this sequence is transcribed, and we call it hereafter SNAI-like (SNAIIL). The change of name and symbol for this gene has been approved by the HGNC (www.gene.ucl.ac.uk/nomenclature/genefamily/snaI).

Comparison of the deduced amino acid sequences of SNAI and SNAI-like (Fig. 1A) shows that the changes are not distributed equally along the whole length of the predicted protein. No changes were observed in the first 9 amino acids (aa)/27 bp, the highly conserved SNAG domain, present in all vertebrate Snai family members (2) and implicated in the repressor activity of these transcription factors (36–38).

We divided the remainder of the SNAI-like protein into the C-terminal zinc finger DNA binding domain and an intermediate putative protein interaction domain. The zinc finger domain showed a 9.9% change in amino acids, whereas the intermediate region had undergone a 22.4% variation (Fig. 1A). This bias toward changes in the intermediate region was also reflected in the nucleotide sequence. The analysis of the substitutions in SNAI-like versus SNAI-coding region is shown in Fig. 1B. The nature of nucleotide substitutions in terms of the ratios of transitions (t)/transversions (T) and of synonymous (S), non-aa-changing/non-synonymous (NS, aa-changing) variations can be used as an indicator of the degree of positive selection within the amino acid sequence (39). We found no difference between the intermediate and C-terminal regions with respect to t/T, but the percentage of NS changes was higher in the intermediate region. These data are indicative of a selective pressure that has led to the conservation of the coding potential of SNAI-like, making it prone to produce an active protein.

The first indication that this sequence is actively transcribed was the identification of two human ESTs derived from the 3′-UTR of SNAI-like. To directly assess the expression of this transcript, we carried out RT-PCR studies in panels of human adult tissues and tumor cell lines using specific pairs of primers. SNAI-like was expressed in a subset of the tissues that normally express SNAI (Fig. 2A) and in all the cell lines analyzed (Fig. 2B). Semi-quantitative analysis by RT-PCR indicates that in the tissues and cell lines where the two genes are expressed, their transcripts are represented at similar levels.

**SNAI-like Is Integrated within an Intron of an Evolutionarily Conserved Novel Gene—**The differential expression of SNAI-like indicates that it contains a functional promoter and that its expression is controlled by cis-regulatory elements either present at the site of genomic insertion or included in the retrotransposed sequence. If the latter were true, such elements might derive from the original SNAI gene or from transposable and/or repeated elements (17). To address these questions, we studied the genomic organization of the region where SNAI-like is inserted.

When searching for known genes in the vicinity of SNAI-like, we found that the MAP2 (microtubule-associated protein
2) gene mapped 78 kb 5′/H11032 and the RPE (ribulose 5-phosphatase 3-epimerase) gene mapped 193 kb 3′/H11032 of SNAIL-like (Fig. 3A).

However, the expression patterns of these genes could not account for the differential expression of SNAIL-like (neural-specific for MAP2 (40) and ubiquitous for RPE (41)). GENSCAN analysis (28) on the 271-kb genomic segment located between these two genes identified a predicted gene that spanned more than 200 kb and coded for a product of 2915 aa, which we have called 2q34-X. The prediction contained part of the coding region of SNAIL-like, lacked the initiation and the stop codons, and is a composite of three independent predicted transcripts, ENST0000236970, ENST0000227845, and ENST0000273046 (www.ensembl.org/), spanning three contiguous bacterial artificial chromosomes (BACs) (accession numbers AC006385, AC006464, and AC007038).

In the search for potential homologues, we found that the C-terminal third of this product corresponds to the KIAA1843 protein, identified in a large scale sequencing and expression study of large cDNAs expressed in the human brain (42). We also found a predicted protein of 3147 aa from C. elegans (F25C8.3) that showed significant similarity to 2q34-X distributed in blocks along the whole length of both products (Fig. 3B).

In Drosophila melanogaster, the predicted gene product CG18437 (252 aa long) matches the N-terminal end of F25C8.3 and 2q34-X proteins. Analysis of the Drosophila genomic segment containing this gene (accession number AE003762) unveiled a putative product of 3044 aa, similar to both human 2q34-X and C. elegans F25C8.3 along its whole length (Fig. 3B). Therefore, we have identified an evolutionary conserved gene family that encodes proteins of 3000 aa, spanning 10 kb in C. elegans, 40 kb in Drosophila, and 200 kb in human. The predicted proteins do not contain any known domain or similarity to other known proteins that might provide us with a clue as to their function.

These data provide evidence that 2q34-X represents a true transcription unit encoding a functional protein. Indeed, when the human predicted protein was used in searches with TBLASTN, we detected multiple matching ESTs distributed over its whole length. Among these, we found evidence for fusion transcripts including 5′ regions of 2q34-X and the majority...
of the coding region of SNAIL-like, similar although not identical to the gene prediction described above. Other ESTs contained exons surrounding the point of insertion of SNAIL-like, but not retrotransposed sequences, pointing to a complex transcriptional profile including both independent and fusion transcripts.

**Comparison of SNAIL-like and 2q34-X**—The possible presence of both independent and fusion transcripts from the 2q34-X and SNAIL-like genes led us to analyze their relative expression patterns. Because of the similarity between SNAIL and SNAIL-like sequences, they cross-hybridize when used as probes in Northern blot analyses. Thus, we selected several cell lines, including MCF7 because it expresses SNAIL-like but not SNAIL. A transcript of the expected size of complete SNAIL-like or SNAIL transcripts (around 2 kb) was detected in MCF7, MDA, and A375P RNA preparations, indicating that at least in the MCF-7 cell line, independent SNAIL-like transcripts are present. In addition, signals for transcripts of more than 6.5 kb were observed in A375P, which may correspond to a fusion transcript with 2q34-X exons (not shown).

To compare the expression of 2q34-X and SNAIL-like by RT-PCR, we designed primers to amplify two regions of the putative 2q34-X protein coding sequence, boxes A and B, that surround the point of insertion of the retrotransposed fragment in human, C. elegans and Drosophila (Fig. 3). These boxes were chosen since similar sequences were found in mouse ESTs and in genomic sequences from the teleost fish Takifugu rubripes and the urochordate Ciona intestinalis, extending the phylogenetic range where this new gene is present (Fig. 4A). We detected Box B transcripts in adult brain and pancreas (Fig. 4B) and in the melanoma A375P and colon carcinoma LoVo cells (Fig. 4C), a subset of those cell lines expressing SNAIL-like. The same results were obtained for the amplification of Box A in all cases (not shown).

To discard the possibility that some SNAIL-like amplifications originated from fusion transcripts, we performed a RT-PCR analysis with primers specific to its 5′-UTR, absent from both the gene prediction and the EST fusion transcripts. The expression profile obtained was identical to that described (Figs. 2, A and B, and 4C), corroborating the existence of independent SNAIL-like transcripts.

**SNAIL-like Can Induce EMT in Epithelial Cells in Culture**—To identify the possible function of SNAIL-like, we overexpressed the coding region of the retrotransposed gene in a cell line (MDCK) and compared its effects with those observed after overexpression of the original SNAIL gene. We have previously shown that stable transfectants of mouse SNAIL in these cells causes a dramatic change in phenotype (epithelial to fibroblastic), mirroring the EMT observed in developing embryos (10).

Parental MDCK cells and mock transfectant cells grow as a polarized monolayer with E-cadherin localized at cell junctions (Fig. 5A). In contrast, both SNAIL and SNAIL-like transfectants adopted a spindle-like shape, down-regulate E-cadherin expression, and no longer established tight cell junctions (Fig. 5A). The loss of E-cadherin was confirmed by RT-PCR analysis (data not shown). Thus, SNAIL-like maintains the capacity to induce EMTs associated with the loss of E-cadherin expression in MDCK cells. This is not a property of particular constitutively expressing clones, since it is also observed in an inducible system. We have used a tamoxifen-inducible system to express SNAIL and SNAIL-like in MCDK cells and found that the transfected cells, which also express EGFP, have lost E-cadherin expression at the cell junctions (Fig. 5B).

To further characterize the effect of SNAIL and SNAIL-like overexpression on MDCK cells, we analyzed their migratory and invasive properties in a wound healing assay with the stable transfectants (Fig. 6). Twelve hours after the incision was made, cultures of mock transfectants showed no signs of wound healing (Fig. 6, A and B), whereas SNAIL and SNAIL-like transfectants invaded the area of the wound after only 6 h (Fig. 6, C–F). The invasiveness of the cells was determined by assaying their capacity to migrate through a collagen matrix. Only cells expressing SNAIL or SNAIL-like were able to go through the collagen gel (Fig. 6, G–I). Thus, SNAIL-like has the capacity to induce a fibroblastic transformation and to render MDCK epithelial cells migratory and invasive.

**SNAIL-like Maintains the Ability to Repress E-cadherin Expression**—To check the ability of SNAIL-like to repress the activity of the E-cadherin promoter, we co-transfected SNAIL- and SNAIL-like vectors with a reporter luciferase construct containing the mouse proximal E-cadherin promoter.
Assay of migration through a collagen matrix (A–F) and invasiveness in cultures using a wound healing assay (G–I). Mock transfectants (Mock)–I–F and SNAI1–L show a fibroblastic phenotype, clear down-regulation of E-cadherin, and an increase and redistribution of vimentin. B, double-labeled images showing E-cadherin (red) and EGFP expression (green) in mock, SNAI1-, and SNAI1L-transfected cells using a tamoxifen-inducible system in transient transfection analysis. EGFP staining depicts transfected cells. Observe that both SNAI1- and SNAI1-like-transfected cells have lost E-cadherin expression after tamoxifen treatment. Tam, 4-OH-tamoxifen.

DISCUSSION

SNAIL-like Is an Encoding Gene Subjected to Positive Selection—Gene duplication is one of the driving forces that model genomes during evolution (43). Retrotransposition has been considered as one of the possible mechanisms responsible for these events (25). However, although examples of reverse transcription and insertion of an mRNA are well documented (17), cases of functional duplicates with proven biological significance are very rare. In this work we show that a retrotransposed copy of human SNAIL previously described as a non-active pseudogene, SNAI1P (15, 16), encodes a fully functional factor that retains some of the key roles in directing cellular phenotype. This is one of the few examples, if not the only one, of a duplicated transcription factor originated by retrotransposition that remains active. Thus, this gene is an additional member of the Snail gene superfamily and, therefore, more properly named as SNAIL-like.

Sequence analysis reveals three pieces of evidence to support this view as follows. (i) SNAIL-like conserves a complete open reading frame. (ii) The sequence similarity to SNAIL extends to the 5′- and 3′-untranslated regions. (iii) The number and nature of nucleotide changes are unequally distributed along the coding region. The retrogene holds more changes in the region responsible for putative interactions with other proteins than in the DNA binding region (the zinc finger domain). In addition, the transactivation SNAG domain (26), fundamental for the repressor activity of Snail family members in several species including humans, is unaltered (36–38). A high degree of similarity is also observed in the UTR sequences, suggesting that these regions may also be functional. This analysis argues in favor of a high positive selection (39, 43) and suggests that this retrogene encodes a functional protein.

Transcriptional Control of SNAIL-like Expression—Confirmation that SNAIL-like is expressed is provided by the existence of ESTs derived from the 3′-UTR of SNAIL-like and the analysis of expression in human adult tissues and cell lines. The parental gene, SNAIL, was expressed in many adult tissues. Because it is known to be expressed in fibroblasts and mesenchymal cells (10, 11, 14) we cannot discard the possibility that the observed expression in some tissues is due to the presence of these cell types. Interestingly, SNAIL-like shows a much more restricted expression in adult tissues, implying a specific transcriptional control, different from that of the original gene. Transcripts were detected in brain and pancreas but not in heart, placenta, and lung, the tissues that were previously analyzed and that led to the idea that this retrogene was a non-expressed pseudogene (15).

Regarding expression in the cell lines, SNAIL-like is expressed in cells derived from different human breast and colon carcinomas and one melanoma, whereas SNAIL shows a more
restricted pattern. Thus, once again, SNAIL-like presents a distinct expression pattern, indicating that promoter and regulatory control elements must drive its expression. Because it is extremely unlikely that a retrogene inserts in the proximity to a promoter, the most simple explanation is that part of the 5′-UTR sequences of the retrotransposed SNAIL-like gene fulfills this function. This seems to be the case since a fragment containing part of the corresponding 5′-UTR sequences of the SNAIL gene shows promoter activity in cotransfection assays in cell culture. This suggests that SNAIL may have two alternative promoters, a more distal one, responsible for the transcript that was the template of the reverse transcription, and a second proximal promoter used by SNAIL-like.

The cis-regulatory elements responsible for the differential expression in human tissues could be present in the genomic region where SNAIL-like was inserted or included in the retrotranscribed sequence (17). We have found that SNAIL-like is integrated in an intron of a novel gene, 2q34-X. Therefore, this indicates that it is located in an active region of the genome, accessible to the transcriptional machinery.

The comparison of the expression of SNAIL, SNAIL-like, and 2q34-X indicated that SNAIL-like and 2q34-X are present in a subset of SNAIL-expressing tissues. This suggests that the expression of SNAIL-like may be driven by regulatory elements shared with or from the 2q34-X gene. However, the presence of SNAIL-like transcripts in all cell lines analyzed regardless of the expression of both SNAIL and 2q34-X indicates that the retrotransposed sequence must have lost repressor elements and/or additional cis-regulatory elements.

Independent SNAIL-like transcripts exist, as confirmed in MCF-7 cells. Interestingly, human ESTs that contain sequences of both genes exist. Indeed, A375 cells, which express both genes as assessed by RT-PCR, reveal a transcript bigger due to the presence of independent transcripts. Therefore, this indicates that the SNAIL-like and the Analysis of SNAIL Expression in Tumors—Having shown that SNAIL-like is widely expressed in different tumor cell lines and some normal adult tissues, several important issues emerge. First of all, its potential to induce a full EMT has to be considered both in physiological and pathological studies. It could be speculated that an increase in the amount of this gene product may render it fully competent to induce the phenotypic changes that accompany the acquisition of invasive properties. Thus, its expression might constitute a susceptibility factor to develop malignancy. On the other hand and in contrast to SNAIL, its transcription does not directly correlate with invasiveness, making it extremely important to differentiate the detection of these two very similar genes in expression studies in human tumor cell lines or tumor biopsies. Although several studies have shown a correlation between SNAIL expression and dedifferentiation in human samples (10–14), others have described SNAIL expression regardless of the level of E-cadherin expression in human tumors (47) or cell lines (48). In these cases, due to sequence similarity and experimental approaches (RT-PCR and Northern, respectively), the failure to detect a correlation may be the result of the inadvertent amplification of SNAIL-like. Indeed, as assessed by RT-PCR, the epithelial breast tumor-derived cell line MCF-7 does not express SNAIL (10) but expresses the retrogene (this work) and shows a positive signal in Northern analysis that was interpreted as expression of the parental SNAIL gene (48). In cell lines, this problem can be avoided by the use of SNAIL-like-specific primers such as those used in this study. In conclusion, we have shown here that SNAIL-like is a new human member of the Snail family that must be taken into consideration for fundamental studies.

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