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# A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene

**(serpins/maspin/plasminogen activator inhibitor type 2)**

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**ABSTRACT** **The squamous cell carcinoma antigen (SCCA) is a member of the ovalbumin family of serine proteinase inhibitors (serpins). A neutral form of the protein is found in normal and some malignant squamous cells, whereas an acidic form is detected exclusively in tumor cells and in the circulation of patients with squamous cell tumors. In this report, we describe the cloning of the SCCA gene from normal genomic DNA. Surprisingly, two genes were found. They were tandemly arrayed and flanked by two other closely related serpins, plasminogen activator inhibitor type 2 (*PAI2*) and maspin at 18q21.3. The genomic structure of the two genes, *SCCA1* and *SCCA2*, was highly conserved. The predicted amino acid sequences were 92% identical and suggested that the neutral form of the protein was encoded by *SCCA1* and the acidic form was encoded by *SCCA2*. Further characterization of the region should determine whether the differential expression of the SCCA genes plays a causal role in development of more aggressive squamous cell carcinomas.**

The squamous cell carcinoma antigen (*SCCA1*) gene is a member of the ovalbumin family of serine proteinase inhibitors (Ov-serpins) (1, 2). The protein was isolated from a metastatic, cervical SCC by Kato and Torigoe (3). By SDS/polyacrylamide gel electrophoresis, the protein appears as a single band with an $M_r$ of ≈45,000 (1). However, isoelectric focusing reveals significant charge heterogeneity with proteins being grouped into a neutral (pI = 6.3–6.6; $M_r$ 42,000–48,000) and an acidic fraction (pI = 5.9–6.2; $M_r$ 42,000–48,000) (4). Monoclonal antibodies prepared against SCCA detect at least seven epitopes common to the neutral and acidic forms of the protein and one unique to the acidic form (5).

SCCA is detected in the superficial and intermediate layers of normal squamous epithelium (6), whereas the mRNA is detected in the basal and subbasal levels (7). The clinical import of SCCA has been as a circulating tumor marker for SCC, especially those of the cervix, head and neck, lung, and esophagus (5). Multiple clinical studies of cervical SCC show that the percentage of patients with elevated circulating levels of SCCA increases from ≈12% at stage 0 to >90% at stage IV (5, 6). Levels fall after tumor resection and rise in ≈90% of the patients with recurrent disease. Similar trends occur in the other types of SCC, with a maximum sensitivity of approximately 60% for lung, 50% for esophageal, and 55% for head and neck tumors (5). Elevated circulating levels of SCCA are also detected in a lower percentage of patients with adenocarcinomas of the uterus, ovary, and lung (6). Interestingly, the neutral form of SCCA is detected in the cytoplasm of normal and some malignant squamous cells, whereas the acidic form is expressed primarily in malignant cells and is the major form found in the plasma of cancer patients (5). Thus, the appearance of the acidic fraction of SCCA is correlated with more aggressive tumors.

In our analysis of chromosomal aberrations involving human chromosome band 18q21 we identified a DNA fragment, A56R (*D18S86*) (8), that contained a 56/57-bp match with the published cDNA sequence of *SCCA* (1). In this report, we showed that A56R contained exon 3 of a new gene, *SCCA2*. This gene was 92% identical to *SCCA1*. *SCCA1* and *SCCA2* were tandemly arrayed and flanked by two other Ov-serpins, plasminogen activator inhibitor type 2 (*PAI2*) and maspin. Finally, the predicted pI values and molecular weights of the cDNAs suggested that the neutral and acidic forms of the SCCA were encoded by *SCCA1* and *SCCA2*, respectively.†

## MATERIALS AND METHODS

**Genomic Cloning.** yB29F7 was obtained from a human genomic yeast artificial chromosome (YAC) library (Washington University, St. Louis) as described (8). A rare-cutting restriction map of the clone has been reported (8). Genomic DNA from yB29F7 was partially digested with *Mbo* I and ligated with the λDASHII vector (Stratagene) by using conventional phage cloning techniques (9). Phage DNA was digested with *Xba* I and the resulting fragments were subcloned into pBluescript plasmid vectors (Stratagene).

**DNA Sequencing.** Plasmids were sequenced by standard dideoxynucleotide chain-termination methods (9) using the Sequenase version 2 sequencing kit (United States Biochemicals). DNA sequence was analyzed by using the Genetics Computer Group software package and the BLAST program (10) from the National Center for Biotechnology Information.

**DNA Blotting.** Human genomic, phage, or plasmid DNA was digested with restriction endonucleases and subjected to agarose gel electrophoresis as described (8). DNA blots were hybridized at high stringency (8) with $^{32}$P-labeled probes generated by random priming or the PCR (9). The 2.5-kb maspin cDNA was a generous gift from Ming Zhang and Ruth Sager (Cancer Genetics, Dana–Farber Cancer Institute, Boston).

**PCR.** PCR assays were performed in 10-μl reactions and analyzed as described (8). The primer sequences for maspin (11), elastase inhibitor (*EI*) (12), and human placental throm-

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Abbreviations: EI, elastase inhibitor; OVA, chicken ovalbumin; PAI2, plasminogen activator inhibitor type 2; PTI, placental thrombin inhibitor; SCC, squamous cell carcinoma; SCCA, SCC antigen; serpin, serine proteinase inhibitor; YAC, yeast artificial chromosome; UTR, untranslated region; $\alpha_1$-AT, $\alpha_1$-antitrypsin; $\alpha_1$-ACT, $\alpha_1$-antichymotrypsin; $\alpha_1$-PI, $\alpha_1$-proteinase inhibitor.

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†The sequences reported in this paper have been deposited in GenBank [accession nos. U19558 (*SCCA1*) and U19569 (*SCCA2*)] and the Genome Data Base [accession no. G00-435-238 (*EI*, *PTI*, and maspin primer sets)].

bin inhibitor (*PTI*) (13) were derived from the published cDNA sequence.

For reverse transcriptase (RT)-PCR, 1–3 μg of total RNA was transcribed using random hexamer and avian myeloblastosis virus reverse transcriptase (9). First-strand cDNA was used in a 10-μl PCR (14) with a primer set capable of discriminating *SCCA2* transcripts from those of *SCCA1* (see Fig. 5). RT-PCR products were subcloned into *Eco*RV-digested pBluescript vectors and sequenced as described (8).

## RESULTS

**Mapping, Cloning, and Sequencing of the SCCA Genes.** Previously, we constructed a 2.5-Mb YAC contig to facilitate the analysis of 18q21 chromosomal aberrations (8). This contig established that the Ov-serpin, *PAI2*, is 600 kb telomeric to the protooncogene, *BCL2* (8) (Fig. 1*A*). Restriction mapping of a YAC clone (yB29F7), which is located between these genes, revealed two bands when the 210-bp A56R genomic marker (*D18S86*) was used as a probe. This result was not due to the partial digestion of genomic DNA or the existence of a similar restriction site within the probe. The presence of two bands in digests of DNA from 10 unrelated individuals suggested that the duplication was not an artifact of YAC cloning (Fig. 2).

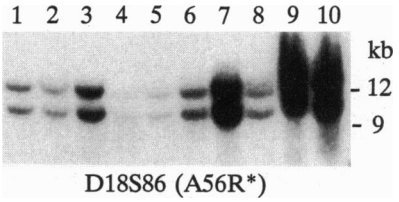


FIG. 2. Duplication of A56R (*D18S86*). Genomic DNA from 10 unrelated individuals (lanes 1–10) was digested with *Eco*RV. The resulting restriction fragments were separated by agarose gel electrophoresis, blotted to reinforced nitrocellulose, hybridized with a $^{32}$P-labeled A56R probe (A56R*), and detected by autoradiography.

A GenBank search using the A56R sequence yielded a 56/57-bp match with the cDNA sequence of the *SCCA1* (1). Appropriate acceptor and donor splice sites flanked the 57-bp sequence. This result suggested that A56R contained an exon of either *SCCA1* or a closely related element. To determine which was the case, we constructed a phage library using yB29F7 genomic DNA. Three overlapping phage clones were isolated using A56R as the probe (Figs. 1*C* and 3). One clone, φA56R-2, contained two different *Xba* I fragments that hybridized with A56R (Fig. 3). The *Xba* I fragments were subcloned into plasmid vectors and analyzed by restriction

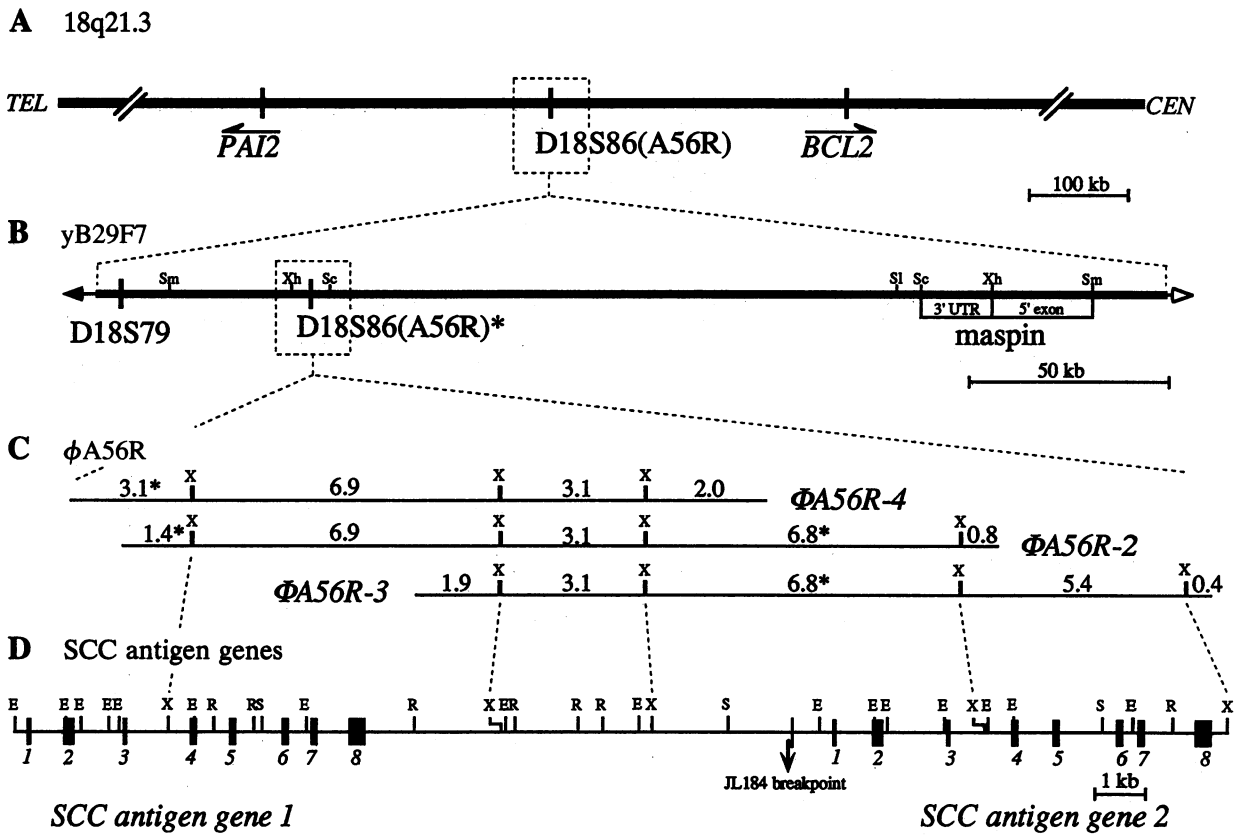


FIG. 1. Mapping and cloning of the serpin genes located at 18q21.3. (*A*) Large-scale genomic map of the region. *D18S86* (A56R) is located ≈300 kb from *BCL2* and *PAI2*. (*B*) Map of yB29F7. The YAC was isolated from a human genomic library using A56R as a probe (8). Maspin was mapped by pulsed-field gel electrophoresis. A probe from the 3′ untranslated region (UTR) hybridized to the left terminal 50-kb *Xho* I fragment but not the 20-kb *Sma* I fragment. A 5′ exon PCR probe hybridized to the left terminal 70-kb *Sac* II fragment but not the terminal *Xho* I fragment. Rare-cutting restriction sites are the same as those reported (8). Sc, *Sac* II; Sl, *Sal* I; Sm, *Sma* I; Xh, *Xho* I. The left (centric) arm of the YAC vector is indicated by the open arrow, and the right (acentric) arm is indicated by the filled arrow. (*C*) A56R phage clones. Three overlapping λ phage clones were isolated from a genomic library prepared from yB29F7 DNA. The clones were oriented using restriction analysis, DNA blotting, and the JL184 breakpoint as a reference (see below). The locations and sizes (kb) of the *Xba* I (X) fragments are indicated. The fragments that contained exon 3 from *SCCA1* and *SCCA2* (marked with an asterisk) hybridized to the 210-bp genomic A56R probe. (*D*) A consensus map of *SCCA1* and *SCCA2*. The *Xba* I fragments from the A56R λ phage clones (*C*) were subcloned into pBluescript, mapped by restriction endonuclease digestion, and partially sequenced to identify intron–exon boundaries of *SCCA1* and *SCCA2*. Of note, A56R contains exon 3 from *SCCA2*. E, *Eco*RI; R, *Eco*RV; S, *Sac* I; X, *Xba* I. The locations of *Xba* I sites, as derived from the phage clones, are indicated by the dashed lines. The orientation of the map is based on a congenital deletion breakpoint (from patient JL184) that occurred between *SCCA1* and *SCCA2*.

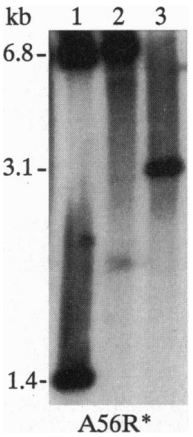


FIG. 3. Restriction mapping of yB29F7 λ phage clones. DNA from three λ phage clones, φA56R-2 (lane 1), φA56R-3 (lane 2), and φA56R-4 (lane 3) was digested with *Xba* I. The fragments were separated by electrophoresis, blotted, and hybridized with a $^{32}$P-labeled A56R probe (A56R*). Only φA56R-2 contained two fragments that hybridized to the probe.

endonuclease digestion and DNA sequencing. The presence of a deletion breakpoint between the duplicated sequences helped provide centromeric–telomeric orientation of the *Xba* I fragments (unpublished data). Sequencing primers derived from the *SCCA1* cDNA sequence were used to confirm the presence of *SCCA1* on the telomeric side of the duplication. Similar to *PAI2* and other Ov-serpins, *SCCA1* contained eight exons, seven introns, and highly conserved intron–exon boundaries (2) (Fig. 1*D*). Genomic sequencing of the DNA located on the centromeric side of the duplication revealed a new gene, *SCCA2*, whose sequence and genomic organization were nearly identical to that of *SCCA1*. Of note, the original sequence of the A56R probe was a perfect match with exon 3 of *SCCA2*.

Alignment of *SCCA2* with *SCCA1* showed that the putative intron–exon boundaries, splice sites, initiation codons, and termination codons were identical. The exon sequences were 98% homologous at the nucleotide level and 92% homologous at the amino acid level. The Kyte–Doolittle hydrophilicity plots and predicted secondary structures of the proteins were similar. However, potentially important differences between the proteins existed at the reactive center loop. The reactive center loop, in part, controls the target-enzyme specificity of the inhibitory serpins (reviewed in refs. 15 and 16). SCCA1 had a Ser-Ser peptide bond at the reactive center ($P_1$–$P_{1'}$), whereas SCCA2 had a Leu-Ser peptide bond at this location. Finally, the deduced amino acid sequence of *SCCA1* predicted a protein with an $M_r$ of 44,500 with a pI = 6.36, whereas that of *SCCA2* predicted an $M_r$ of 44,900 with a pI = 5.8.

The amino acid sequences of SCCA1 and SCCA2 were compared with other Ov-serpins, including EI (12), PTI (13), PAI2 (17), chicken ovalbumin (OVA) (18), and maspin (11) (Fig. 4). Relative to either SCCA1 or SCCA2, EI, PTI, PAI2, OVA, and maspin were 52%, 48%, 44%, 42%, and 35% identical, respectively. In comparison to other members of the serpin superfamily, SCCA1 and SCCA2 were 41%, 35%, 32%, and 31% identical to antithrombin III, $\alpha_1$-proteinase inhibitor [$\alpha_1$-PI ($\alpha$1-antitrypsin)], $\alpha_1$-antichymotrypsin ($\alpha_1$-ACT), and plasminogen activator inhibitor type 1, respectively.

Unlike other members of the serpin superfamily, the Ov-serpins, including SCCA1 and SCCA2, lacked N- and C-terminal extensions and a cleavable hydrophobic signal sequence (2) (Fig. 4). However, like other Ov-serpins (2), these proteins contained an internal hydrophic sequence, which may facilitate secretion, located near the N terminus (Fig. 4).

All members of the serpin family share a highly ordered tertiary structure that consists of nine $\alpha$-helices and three $\beta$-sheets (15, 16). For inhibitory-type serpins, at least 51 residues that are either internal or on surface niches (15) and six residues in the hinge region ($P_{14}$ and $P_{12}$–$P_8$ on the N-terminal side of the reactive center loop) are well conserved (15, 19). SCCA1 and SCCA2 maintained identical or conserved residues at 49/51 positions. The two nonconserved changes were located at the C terminus and were similar to those present in other Ov-serpins (2) (Fig. 4). The hinge region residues located at $P_{12}$–$P_8$ were rich in alanines and conserved in both proteins (Fig. 4).

**Transcription of *SCCA2*.** RT-PCR was used to determine whether *SCCA2* was transcribed. A primer set capable of amplifying the entire coding sequence of *SCCA2* but not that of *SCCA1* was used in a PCR assay with a first-strand cDNA prepared from several different cell lines and human placenta. Abundant PCR product was detected only when cDNA prepared from a head and neck SCC cell line served as the template (Fig. 5). This amplified DNA was subcloned into a plasmid vector and six individual clones were sequenced. In all cases, the sequence of the PCR products matched that predicted for the coding sequence of the *SCCA2* exons (not shown).

**A Serpin Gene Family Located at 18q21.3.** The presence of three closely linked Ov-serpins within 18q21.3 (*PAI2*, *SCCA1*, and *SCCA2*) prompted us to consider whether other family members were nearby. We prepared PCR primer sets for the 3′ UTR of *EI*, *PTI*, and maspin. By using the NIGMS monochromosomal somatic cell hybrid panel (20), *EI* and *PTI* mapped to chromosome 6 and maspin mapped to chromosome 18 (unpublished data). Maspin was localized to band 18q21.3 using DNA from a chromosome 18 deletion panel of somatic cell hybrids (21) (not shown). Finally, maspin was sublocalized to yB29F7, the same YAC clone that contained *SCCA1* and *SCCA2*, by the PCR (Fig. 6) and pulsed-field gel electrophoresis (Fig. 1*B*). From this analysis, we concluded that at least four Ov-serpins resided within a 300-kb region of 18q21.3. Their telomeric to centromeric order was *PAI2*, *SCCA1*, *SCCA2*, and maspin. Using a PCR-based approach, similar conclusions were reached by Sager *et al.* (22). The transcriptional orientation of *SCCA1* and *SCCA2* was telomeric to centromeric and opposite to that of *PAI2* and maspin.

## DISCUSSION

SCCA is a major component of the TA-4 antigen complex that was initially isolated from a metastatic cervical SCC (1). Subsequently, the protein was found to be a serpin that exists in a neutral (pI > 6.2) and an acidic (pI < 6.2) form of similar molecular weights (4). Whereas the neutral form is found in the cytoplasm of normal and some malignant squamous cells, the acidic form is found predominantly in the cytoplasm of the tumor cells (5). The acidic form also accounts for the increased levels of circulating SCCA detected in cancer patients (5). Although alternative splicing or posttranslational modifications such as N-glycosylation could account for the two forms of the protein, data from this study suggested that they were encoded by two separate, but highly homologous (92% at the amino acid level), genes. The deduced pI values and molecular weights of the translation products suggested that the neutral form was encoded by the original SCCA gene (*SCCA1*) and the acidic form was encoded by a new gene, *SCCA2*. The detection of abundant *SCCA2* transcripts in a SCC cell line supported this hypothesis. Near-identical genomic organization (including most restriction sites and intron–exon boundaries), highly conserved amino acid sequence, similarly sized transcripts on Northern blots, and the presence of seven of eight shared antigenic determinants probably account for the difficulty, in previous studies, in detecting the presence of both genes.

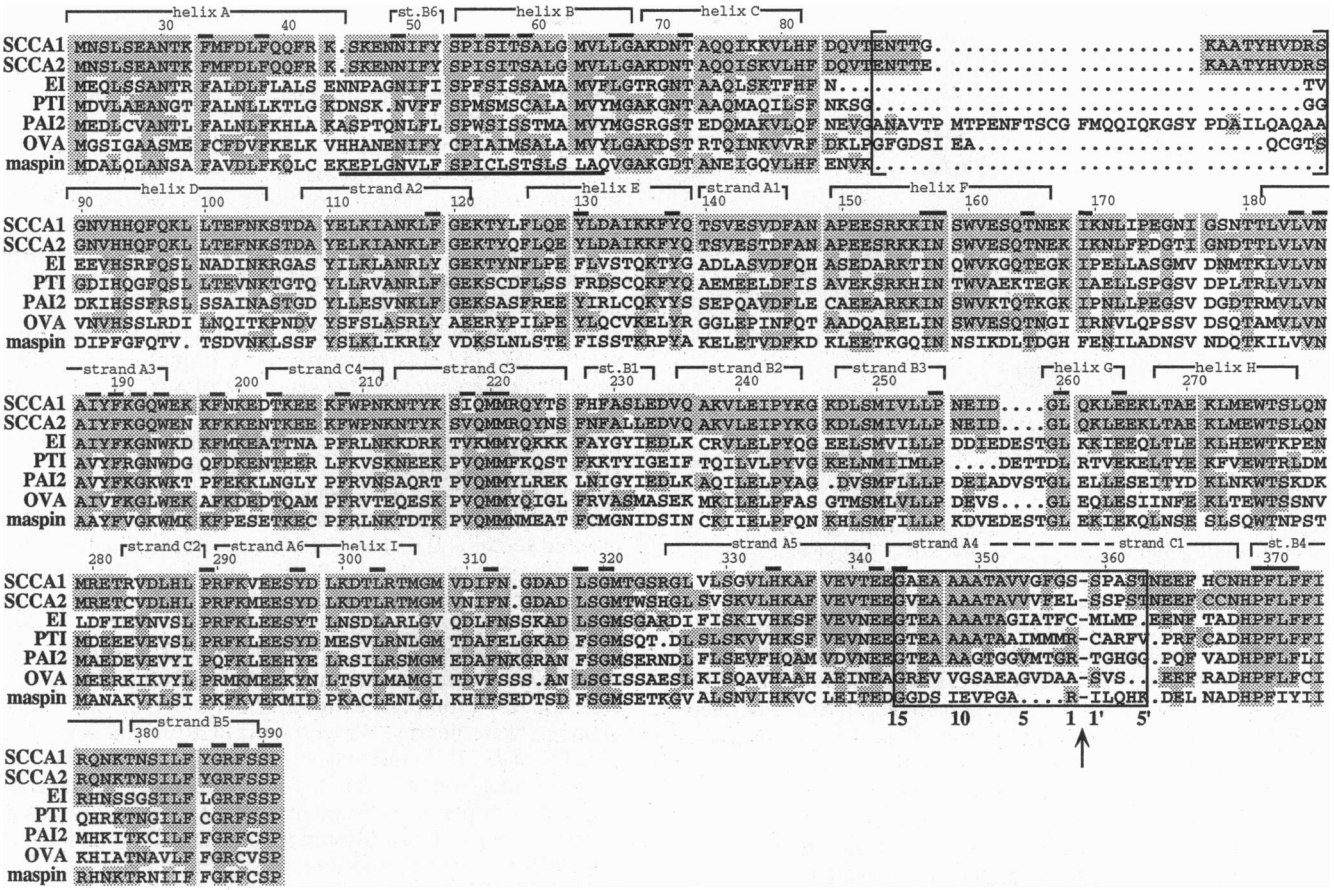


FIG. 4. Amino acid comparison of the Ov-serpin family. The sequences were aligned using the Genetics Computer Group PILEUP program and manually adjusted to give the best fit relative to the canonical $\alpha_1$-antitrypsin ($\alpha_1$-AT) sequence (15). Consensus sequences are shaded and proteins are listed by decreasing homology to *SCCA1*. The numbering of Ov-serpins begins with residue 23 of $\alpha_1$-AT and structural motifs located above the sequence are based on the crystal structure of $\alpha_1$-AT. Sequences in the large bracket represent the variable interhelical region. The internal secretion signal is underlined and the reactive center loop is boxed. The scissile bond is marked with an arrow. Residues in the reactive center are numbered $P_{15}$–$P_{5'}$ (N terminus to C terminus). For inhibitory-type serpins, the conserved amino acid residues at the hinge region are T, A, A, A, A, and T at $P_{14}$, $P_{12}$, $P_{11}$, $P_{10}$, $P_9$, and $P_8$, respectively. The 51 residues conserved in the serpin superfamily are overscored. Ov-serpins have substituted Y, F, or C for V at position 388 and S for N at 390 (2).

The results of this study also showed that *SCCA1* and *SCCA2* were closely linked, tandemly arrayed, and flanked by two other closely related members of the Ov-serpin family, *PAI2* and maspin. All four serpins were located within a 300-kb region of 18q21.3. Two other closely related Ov-serpins, *EI* and *PTI*, mapped to chromosome 6. A different family of serpins containing $\alpha_1$-PI, $\alpha_1$-ACT, corticosteroid binding globulin (CBG), and protein C inhibitor map to a 280-kb region within 14q32.1 (23). Although genes in this cluster are highly homologous, they have evolved different functions. For example, human $\alpha_1$-ACT inhibits cathepsin G and CBG is a noninhibitory-type serpin that serves as a transport protein for cortisol (reviewed in ref. 16).

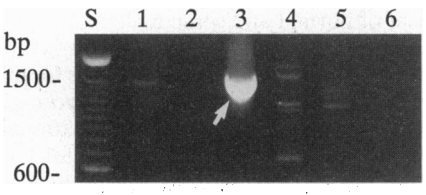


FIG. 5. Transcription of *SCCA2*. Primers capable of amplifying the 1285-bp putative coding sequence of *SCCA2* (5′ UTR sense primer, 5′-CCTCTGCTTCCTCTAGGAAC-3′; 3′ UTR antisense primer, 5′-TCAGTTTACCAGAACACCTCT-3′) were used in a PCR assay with human genomic DNA (lane 1) or first-strand cDNA from REH cells (a human B-cell lymphoma cell line) (lane 2), SQ208 cells (a head and neck SCC cell line) (lane 3), HeLa cells (lane 4), and human placenta (lane 5). Lane 6 is a no DNA control. PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. Standard (S), 100-bp ladder. The DNA from the large band in lane 3 (arrow) was eluted from the gel, cloned, and sequenced. Six subclones were sequenced and all were found to be *SCCA2*. The identity of the spurious bands in the other lanes was not determined.

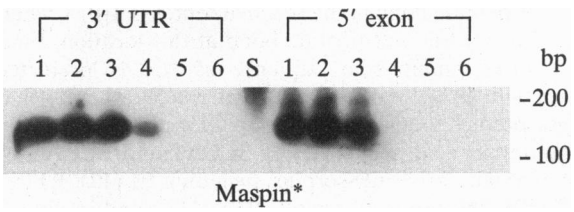


FIG. 6. Localization of maspin to the 18q21.3 YAC, yB29F7. (*Left*) Primers that amplify 164 bp of the 3′ UTR (sense, 5′-GCATAGCCCATGTTAAGTCC-3′; antisense, 5′-AGGTCTATCTGTGTGAAGGC-3′) and (*Right*) 135 bp of a 5′ ATG-containing exon (sense, 5′-AAATTCGGCTTTTGCCGTTG-3′; antisense, 5′-TTTGCAGTGTCACCTTTAGC-3′) of the maspin gene were used in a PCR containing HHW324 DNA (a hamster–human monochromosomal 18 hybrid) (lanes 1), human genomic DNA (lanes 2), yB29F7 DNA (lanes 3), Chinese hamster ovary (CHO) DNA (lanes 4), y302F10 (anonymous human YAC DNA control) (lanes 5), and no DNA (lanes 6). PCR products were separated by agarose gel electrophoresis, blotted, and hybridized with a $^{32}$P-labeled maspin cDNA probe (Maspin*) that contains the ATG through the 3′ UTR. The faint hybridization in the CHO lane (*Left*) may reflect conservation of sequence between species.

Interestingly, the *BCL2* gene is closely linked to the serpin cluster at 18q21.3 (8) and the immunoglobulin heavy chain locus is closely linked to the serpin cluster at 14q32.1 (23). In ≈80% of follicular B-cell lymphomas, the *BCL2* gene is translocated into the immunoglobulin heavy chain locus (24). Perhaps, nonhomologous pairing between members of the two serpin gene clusters facilitates this interchromosomal recombination.

In the mouse, at least four $\alpha_1$-PI-like genes are clustered in the *Spi-1* locus on chromosome 12 (25). The *Spi-1* locus is syntenic with the cluster of serpins on human chromosome 14. A second closely linked locus on chromosome 12, *Spi-2*, contains at least 12 $\alpha_1$-ACT-like genes, including the trypsin inhibitor, contrapsin (25, 26). Considering that *SCCA1*, *SCCA2*, and maspin are located in the 600-kb interval between human *BCL2* and *PAI2* and that *pai2* and *bcl2* are closely linked on mouse chromosome 1, we might predict the presence of another *Spi* locus ≈45 centimorgans from the centromere.

The amino acid sequences of SCCA1 and SCCA2 suggest that these proteins are inhibitory-type serpins. By comparing their reactive centers to those of other inhibitory serpins, we can obtain clues as to the type of enzymes they inhibit. The $P_1$–$P_{1'}$ reactive center of SCCA1 (Ser-Ser) is similar to those of bovine $\alpha_1$-ACT (Ser-Ser) (27) and murine $\alpha_1$-PI (Tyr-Ser) (28). Although the target enzyme for bovine $\alpha_1$-ACT has yet to be identified, mouse $\alpha_1$-PI inhibits elastase (28). The $P_1$–$P_{1'}$ of SCCA2 (Leu-Ser) is similar to that of human $\alpha_1$-ACT (Leu-Ser) and heparin cofactor II (Leu-Ser). The target proteinases of these inhibitors are cathepsin G and thrombin, respectively (16). PAI2 (Arg-Thr) and maspin (Arg-Ile) have similar reactive centers. PAI2 inhibits trypsin-like proteinases such as urokinase-type plasminogen activator (17). However, the absence of a conserved hinge region at the N-terminal side of the reactive center loop and the presence of a motif that binds thymosin $\beta_4$ have prompted some to consider maspin as a ligand-binding, noninhibitory-type serpin (29). Thus, the functions of the Ov-serpins at 18q21.3 appear to have diverged in a fashion analogous to that observed in other serpin clusters.

Chromosomal aberrations involving human chromosomal band 18q21 are associated with several forms of human cancer. These include follicular B-cell lymphoma, osteosarcoma, and carcinomas of the colon, head and neck, ovary, kidney, breast, and bladder (reviewed in ref. 30). These aberrations lead to gain- or loss-of-function mutations in genes that presumably participate in the disease process. Although candidate genes have been identified in follicular B-cell lymphoma (*BCL2*) and colorectal carcinoma (*DCC*), other candidate genes have yet to be identified. Serpins can suppress and enhance tumor growth and spread. For example, a transfected maspin gene can suppress the ability of a mammary carcinoma line to grow and metastasize in nude mice (11), while other serpins may protect cells from activating a programed cell death pathway(s) (31, 32). Thus, the Ov-serpins at 18q21.3 should be added to the group of candidate genes to consider when evaluating the molecular consequences of chromosome 18 rearrangements in some forms of human cancer.

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