Biochemical and Biophysical Research Communications **264,** 299–304 (1999)
Article ID bbrc.1999.1453, available online at http://www.idealibrary.com on IDEAL®

Identification and cDNA Cloning of *headpin,* a Novel Differentially Expressed Serpin That Maps to Chromosome 18q

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Received August 21, 1999

Differential display was used to identify a novel serpin (*headpin*) underexpressed in squamous cell cancers of the oral cavity. *Headpin* cDNA encoding a complete open reading frame was cloned and sequenced. *Headpin* is expressed in normal oral mucosal tissue, skin, and cultured keratinocytes. Using Northern analysis and relative reverse-transcription polymerase chain reaction (relative RT-PCR), downregulation of *headpin* mRNA expression was demonstrated in oral cavity squamous carcinomas. Northern blot analysis identified a 3.3-kb *headpin* mRNA transcript. Headpin is a 391-amino-acid protein with a theoretical molecular weight of 44 kDa. Hinge region homology at the reactive site loop suggests that *headpin* belongs to the inhibitory class of serine protease inhibitors. *Headpin* was mapped to 18q21.3/18q22. This region includes the ovalbumin serpins (ov-serpins) *maspin, SCCA1, SCCA2,* and *PAI-2.* Furthermore, 18q is recognized as a region for frequent loss of heterozygosity (LOH) in head and neck cancer and other malignancies. © 1999 Academic Press

Serine proteinase inhibitors (serpins) comprise a diverse group of proteins that form a superfamily that already includes more than 100 members from such diverse organisms as viruses, plants and humans. Serpins have evolved over 500 million years and diverged phylogenetically into proteins with inhibitory function and non-inhibitory function (1). Noninhibitory serpins such as ovalbumin lack protease inhibitory activity (2). Whether or not a serpin possesses inhibitory function depends strongly on the consensus sequence located in the hinge region of the reactive site loop near the carboxy-terminus of the coding region. The primary function of serpin family members appears to be neutralizing overexpressed serine proteinase activity (3). Serpins play a role in extracellular matrix remodeling, modulation of inflammatory response and cell migration (3). In humans, serpins may be present both intracellularly and extracellularly (4). Serpin families tend to cluster at specific chromosomal locations. The ov-serpin family clusters at 18q21.3 and includes *maspin*, *SCCA1*, *SCCA2* and *PAI-2* (4, 5). The 18q21-23 chromosomal region exhibits frequent site of loss of heterozygosity in head and neck carcinomas and other solid malignancies (6–8). Interestingly, this is also the locus of *maspin*, which behaves as a tumor suppressor (9).

Using differential display reverse transcription-based PCR (DDRT-PCR) to compare normal squamous epithelium and squamous cell carcinomas of the oral cavity we identified a differentially expressed novel gene fragment with homology to the serpin family. Through a combination of 5′ rapid amplification of cDNA ends (5′ RACE) analysis and cDNA library screening the entire open reading frame of the differentially expressed gene was determined to encode a novel, previously uncharacterized member of the serpin family, which we have designated *headpin* (head and neck serpin). We have cloned, sequenced and examined expression patterns in normal tissues of this new member of the serpin superfamily and mapped it to a locus within the serpin cluster on chromosome 18q. Furthermore, we have demonstrated reduced *headpin* expression in oral cavity carcinomas compared to site-matched normals by relative RT-PCR. Based on a combination of characteristics, we believe that *headpin* is a

This work was supported in part by Grant R29 DE11689-01A1 from the National Institute of Health First Investigator Award (G.L.C.), Grant 1P50DE11906-01, Oral Cancer Research Center of Excellence Award of the National Institute of Dental and Craniofacial Research (G.L.C.), and Grant NIH-NCI-CA 16672 from the Cancer Center Support Grant.

The GenBank accession number given to headpin is AF169949.

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0006-291X/99 $30.00

member of the ovalbumin family of the inhibitory class of serpins with as yet undetermined function.

MATERIALS AND METHODS

Materials. All oligonucleotide primers were synthesized and cartridge purified by Genosys Biotechnologies, Inc. (The Woodlands, TX). Superscript II (GibcoBRL, Gaithersburg, MD) reverse transcriptase was used in all reverse transcription reactions. Sequencing of differential display products was performed by using the Sequenase Kit (Amersham, Piscataway, NJ). Sequencing of cDNA clones for sequence establishment and confirmation of headpin was performed by the MDACC DNA core facility. Differential display was performed using the Differential Display Kit (Display Systems Biotech, Vista, CA).

Cloning and sequence analysis of headpin. The differential display fragment corresponding to *headpin* was sequenced and GenBank was searched using the BLASTN program at the National Center for Biotechnology Information (NCBI) in the non-redundant database (http://www.ncbi.nlm.nih.gov). The GenBank search matched our fragment to 40 bp of a partial cDNA fragment HUR 7 (Accession No. X98307). A limited 5′ RACE (Boehringer-Mannheim, Indianapolis, IN) was performed using nested anti-sense primers to the combined *headpin-HUR 7* sequence. Subsequently, a lambda gt11 phage human foreskin keratinocyte cDNA library (Clontech, Palo Alto, CA) was screened using the extended *headpin* cDNA as a probe. The positive plaques were selected and their cDNA were isolated, subcloned into a Topo TA Cloning kit system (Invitrogen, Carlsbad, CA), and sequenced. A full-length cDNA fragment covering the open reading frame was also generated by polymerase chain reaction (PCR) and eight independent clones confirmed the sequence of *headpin*.

Relative RT-PCR. Samples were obtained from normal and tumor site-matched biopsy tissue from five patients with squamous cell carcinomas. Tissue acquisition was obtained for a protocol of tumor banking which is approved by the institution's surveillance committee. The tissues were homogenized and the total RNA was extracted using Trizol Reagent (GibcoBRL). In addition, total RNA was also extracted from seven established head and neck tumor cell lines and primary cultures of normal oral epithelial cells. RT reactions of the biopsy tissue specimens were performed in duplicate using 2 μg of total RNA. Those of the cell lines were performed in single reactions using 2 μg of total RNA.

Probes specific for the 3′ end of *headpin* were chosen that had the least nucleotide identity to other closely-related serpins. The *headpin* probe sense primer was 5′-GTCCAGGGCATATGGAAGAA-3′ and the antisense was 5′-GGGATGATTGCAGTG-AACATT-3′. Prior to relative RT-PCR, a PCR titration using two dilutions (1:20, 1:200) of our highest-expression biopsy specimen was performed to establish the PCR conditions that were in the linear range for detecting *headpin*. After the appropriate PCR cycle number was determined, we performed relative RT-PCR on duplicate RT reactions of biopsy specimen total RNA under the following PCR conditions; a hot start at 94°C for 30 s followed by 28 cycles of 94°C × 30 s, 59°C × 40 s and 72°C for 1 min and a 7-min extension at 72°C. The 18S competimer: primer system (Ambion, Austin, TX) designed to PCR amplify a fragment of 18S RNA was used as an internal control for normalization of PCRs. At a competimer:primer ratio of 8:2, the 18S product was also in linear range. Relative RT-PCR products were resolved on 6% polyacrylamide gels in 0.5% Tris/Acetic acid/EDTA buffer run at 18 W. Stoffel *Taq* polymerase (Perkin–Elmer, Norwalk, CT) was used for the relative RT-PCR.

Northern blot analysis. A human multiple tissue Northern blot filter containing 2 μg of poly $(A)^+$ RNA was purchased from Clontech and hybridized in Rapidhyb buffer (Amersham) at 65°C for 2.5 h. with *headpin* cDNA (nt 815–1107) radiolabeled with [α-^{32}P]dCTP. The filter was washed in 2× SSC/0.1% SDS at room temperature and then washed twice in 1× SCC/0.1% SDS at 65°C. An additional Northern blot was done to confirm transcript size and compare normal and tumor transcript expression. 5 μg of total RNA from a normal and patient matched squamous cell carcinoma was loaded onto a denatured polyacrylamide gel and run on a horizontal gel apparatus. The RNA was transferred to a nylon membrane overnight and the membrane was hybridized to the *headpin* probe.

Radiation hybrid (RH) mapping of headpin. To determine chromosome localization of the *headpin* gene, we performed RH mapping using the GeneBridge 4 RH panel (Research Genetics, Huntsville AL) with a set of primers located within the putative exon 8 (5). Appropriate positive (donor) and negative (receptor) controls were performed before performing the PCR-based assay and submitting the results to the Whitehead genome server at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl.

RESULTS AND DISCUSSION

Isolation and characterization of headpin cDNA. We analyzed 5 normal and 5 tumor patient-matched specimens from oral cavity squamous epithelium using DDRT-PCR. After subcloning and sequencing one of the differentially expressed fragments, we conducted a search of all NCBI/NIH nucleotide data banks. The differentially displayed fragment matched 40 bp at the 5′ end of a 412-bp partial sequence called *HUR 7* in the non-redundant database, which encodes an open reading frame, stop site and putative poly-adenylation tail (10). After performing a limited 5′ RACE to extend the open reading frame, we screened a human keratinocyte cDNA using a hybrid *headpin-HUR 7* probe and obtained the remaining *headpin* sequence from a series of overlapping clones. *Headpin* diverges from *HUR 7* at the 3′ end and contains a considerably longer open reading frame.

The coding sequence of the 1173 nucleotide *headpin* cDNA and corresponding amino acid sequence encoded therein are shown in Fig. 1. The predicted start methionine is located 30 bp downstream from the 5′ end of the cDNA fragment and is included in the single open reading frame of 391 amino acids. Headpin has a predicted M_r of 44 kDa and a calculated p*I* of 5.5.

The reactive site loop (RSL) located approximately 30–50 amino acids from the carboxy-terminus is a variable region among serpins and is the domain which binds to the active site of serine proteinases (3). Situated at the amino-terminus of the RSL is a highly conserved region known as the hinge region (P15–P9) (P-numbering system according to Schechter and Berger) which predicts whether or not a serpin behaves in an inhibitory capacity (11). The consensus sequence for the hinge region that predicts whether a serpin belongs to the inhibitory class is GTXAAAAT where threonines are located at P14 and P8 (12). The predicted hinge region sequence for headpin is GTEAAAAT (Fig. 2) and thus adheres strictly to the consensus sequence suggesting that it is an inhibitory serpin. Mutations in this region dramatically affect an inhibitors ability to function (3). Another region in the

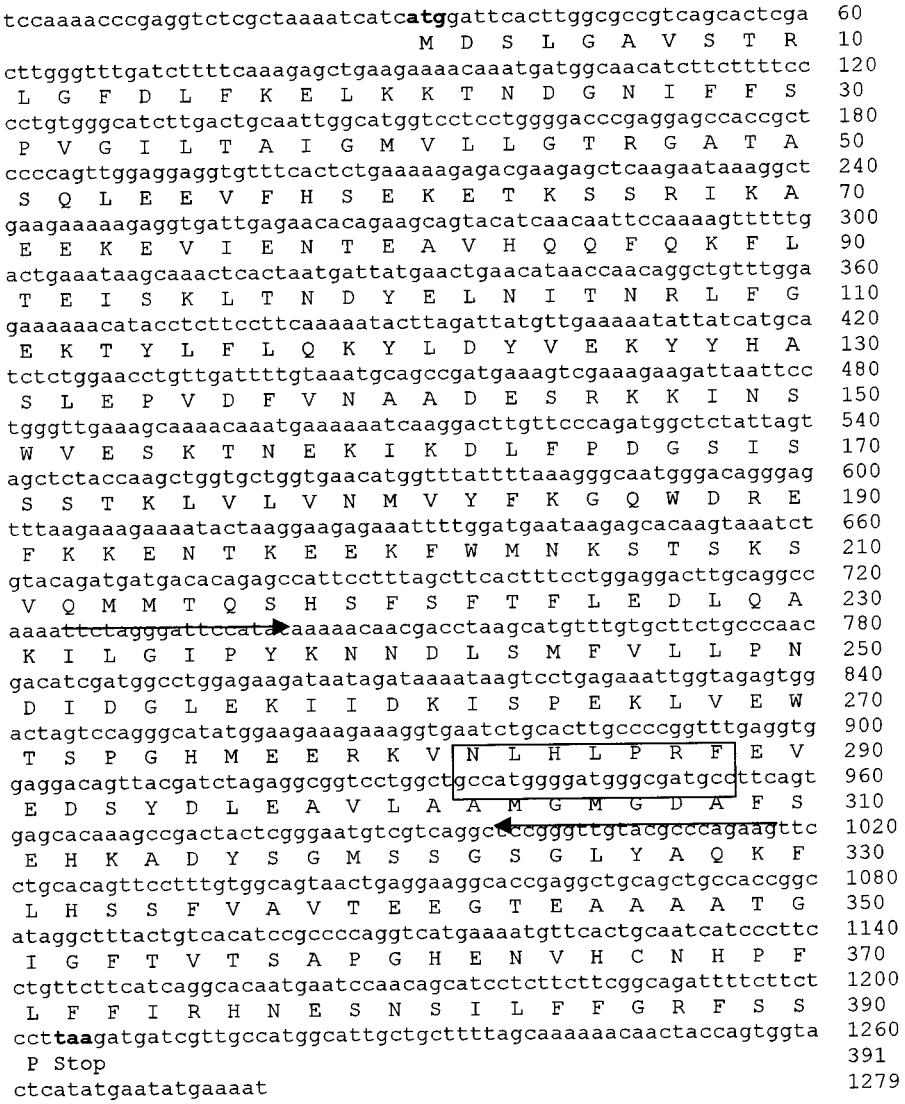

FIG. 1. cDNA sequence and deduced amino acids of headpin. The box indicates the hinge region of the reactive site loop. The position of the primer sets used for both the relative RT-PCR and synthesis of the probe for the Northern blots are indicated by arrows below the nucleotide sequence.

RSL (known as the scissile bond site) located at the putative P1–P1′ of the inhibitory serpins involves the highly specific binding of the target proteinase. This specific covalent binding initiates a series of conformational changes that render the proteinase inactive (13). The scissile bond site of *headpin* is threonine and serine at P1 and P1′, respectively (Fig. 2). Among inhibitory serpins, serine often occupies the P1′ position (12).

Homology of headpin with ov-serpins. A comparison of amino acid sequences of *headpin* and other serpins using the Clustal W protein comparison site at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/) revealed 59, 58, and 42% overall sequence identity with other closely related ov-serpins *SCCA1*,

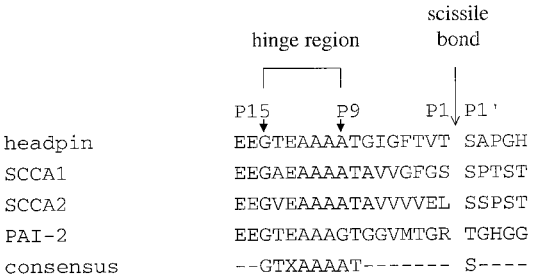

FIG. 2. Comparison of reactive site loop sequences in headpin with other ov-serpins. Consensus hinge region is identical to headpin. SCCA1, squamous cell antigen 1; SCCA2, squamous cell antigen 2; PAI2, plasminogen activator inhibitor-2. The numbers of the amino acids correspond to the nomenclature of Schechter and Berger (11). The location of the scissile bond and the conserved P1′ serine are indicated.

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headpin          MDSLGAVSTRLGFDLFKELKKTN-DGNIFFSPVGILTAIGMVLLGTRGATASQLEEVFHS
headpin^var      MDSLGAVSTRLGFDLFKELKKTN-DGNIFFSPVGILTAIGMVLLGTRGATASQLEEVFHS
HUR7             ------------------------------------------------------------
SCCA1            MNSLSEANTKFMFDLFQQFRKSK-ENNIFYSPISITSALGMVLLGAKDNTAQQIKKVLHF
SCCA2            MNSLSEANTKFMFDLFQQFRKSK-ENNIFYSPISITSALGMVLLGAKDNTAQQISKVLHF
PAI2             MEDLCVANTLFALNLFKHLAKASPTQNLFLSPWSISSTMAMVYMGSRGSTEDQMAKVLQF

headpin          EKE---------TKSSRIKAEEKEVI-----------ENTEAVHQQFQKFLTEISKLTND
headpin^var      EKE---------TKSSRIKAEEKEVVRIKAEGKEI--ENTEAVHQQFQKFLTEISKLTND
HUR7             ------------------------------------------------------------
SCCA1            DQV---------TENTTGKAATYHVDR------------SGNVHHQFQKLLTEFNKSTDA
SCCA2            DQV---------TENTTEKAATYHVDR------------SGNVHHQFQKLLTEFNKSTDA
PAI2             NEVGANAVTPMTPENFTSCGFMQQIQKGSYPDAILQAQAADKIHSSFRSLSSAINASTGD

headpin          YELNITNRLFGEKTYLFLQKYLDYVEKYYHASLEPVDFVNAADESRKKINSWVESKTNEK
headpin^var      YELNITNRLFGEKTYLFLQKYLDYVEKYYHASLEPVDFVNAADESRKKINSWVESKTNEK
HUR7             ------------------------------------------------------------
SCCA1            YELKIANKLFGEKTYLFLQEYLDAIKKFYQTSVESVDFANAPEESRKKINSWVESQTNEK
SCCA2            YELKIANKLFGEKTYQFLQEYLDAIKKFYQTSVESTDFANAPEESRKKINSWVESQTNEK
PAI2             YLLESVNKLFGEKSASFREEYIRLCQKYYSSEPQAVDFLECAEEARKKINSWVKTQTKGK

headpin          IKDLFPDGSISSSTKLVLVNMVYFKGQWDREFKKENTKEEKFWMNKSTSKSVQMMTQSHS
headpin^var      IKDLFPDGSISSSTKLVLVNMVYFKGQWDREFKKENTKEEKFWMNKSTSKSVQMMTQSHS
HUR7             ------------------------------------------------------------
SCCA1            IKNLIPEGNIGSNTTLVLVNAIYFKGQWEKKFNKEDTKEEKFWPNKNTYKSIQMMRQYTS
SCCA2            IKNLFPDGTIGNDTTLVLVNAIYFKGQWENKFKKENTKEEKFWPNKNTYKSVQMMRQYNS
PAI2             IPNLLPEGSVDGDTRMVLVNAVYFKGKWKTPFEKKLNGLYPFRVNSAQRTPVQMMYLREK

headpin          FSFTFLEDLQAKILGIPYKNNDLSMFVLLPNDID----GLEKIIDKISPEKLVEWTSPGH
headpin^var      FSFTFLEDLQAKILGIPYKNNDLSMFVLLPNDID----GLEKIIDKISPEKLVEWTSPGH
HUR7             -----LEDLQAKILGIPYKNNDLSMFVLLPNDID----GLEKVNAYTSLFFLSFPKAFCL
SCCA1            FHFASLEDVQAKVLEIPYKGKDLSMIVLLPNEID----GLQKLEEKLTAEKLMEWTSLQN
SCCA2            FNFALLEDVQAKVLEIPYKGKDLSMIVLLPNEID----GLQKLEEKLTAEKLMEWTSLQN
PAI2             LNIGYIEDLKAQILELPYAG-DVSMFLLLPDEIADVSTGLELLESEITYDKLNKWTSKDK

headpin          MEERKVNLHLPRFEVEDSYDLEAVLAAMGMGDAFSEHKADYSGMSSGSGLYAQKFLHSSF
headpin^var      MEERKVNLHLPRFEVEDSYDLEAVLAAMGMGDAFSEHKADYSGMSSGSGLYAQKFLHSSF
HUR7             RASE*--------------------------------------------------------
SCCA1            MRETRVDLHLPRFKVEESYDLKDTLRTMGMVDIFNGD-ADLSGMTGSRGLVLSGVLHKAF
SCCA2            MRETCVDLHLPRFKMEESYDLKDTLRTMGMVNIFNGD-ADLSGMTWSHGLSVSKVLHKAF
PAI2             MAEDEVEVYIPQFKLEEHYELRSILRSMGMEDAFNKGRANFSGMSERNDLFLSEVFHQAM

headpin          VAVTEEGTEAAAATG-IGFTVTSAPGHENVHCNHPFLFFIRHNESNSILFFGRFSSP* 391
headpin^var      VAVTEEGTEAAAATG-IGFTVTSAPGHENVHCNHPFLFFIRHNESNSILFFGRFSSP* 400
HUR7             ------------------------------------------------------------  55
SCCA1            VEVTEEGAEAAAATAVVGFGSSPTSTNEEFHCNHPFLFFIRQNKTNSILFYGRFSSP* 390
SCCA2            VEVTEEGVEAAAATAVVVVELSSPSTNEEFCCNHPFLFFIRQNKTNSILFYGRFSSP* 390
PAI2             VDVNEEGTEAAAGTGGVMTGRTG-HGGPQFVADHPFLFLIMHKITKCILFFGRFCSP* 415
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FIG. 3. Alignment of the headpin amino acid sequence with other serpins. Asterisk (*) indicates the stop codon. The sequences for the *headpin* variant (headpin^{var}) and *HUR7* are also indicated.

SCCA2, and *PAI-2*, respectively (Fig. 3). Interestingly, we also cloned a variant of *headpin* that contains the amino acid insert VRIKAEGKE located between amino acids 75 and 76. This insertion lies within helix C of the protein crystal structure of serpins and would be expected to affect the highly variable interhelical region between helices C and D (2). The insertion is completely in frame and has been confirmed in full-length clones generated by RT-PCR. *HUR 7* does not conform to the standard serpin pattern because it lacks a hinge region, reactive site loop and prematurely truncates (Fig. 3). Many members of the ov-serpin family rely on an internal signal peptide rather than traditional signal sequences that allow individual serpins to be either secreted or remain within the cytoplasm. As with all ov-serpin family members, *headpin* has a penultimate serine residue rather than an asparagine and lacks a cleavable hydrophobic signal sequence found in the larger serpin superfamily (2, 4). Based on the features such as amino acid identity and structural lack of cleavable signal sequence, *headpin* is very likely a new member of the ov-serpin family (2).

Northern blot and multiple tissue northern (MTN) blot analysis. Northern blot analysis was performed to establish the length of *headpin* RNA transcript. Total RNA from normal and tumor biopsy specimens derived from the oral cavity were hybridized to a *headpin* specific cDNA probe. A 3.3-kb mRNA band was

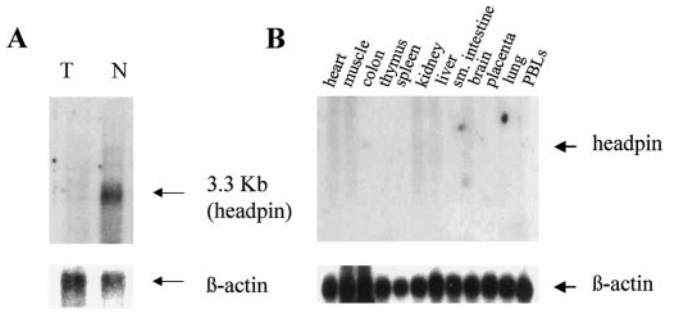

FIG. 4. Detection of the *headpin* transcript in normal squamous epithelium. (A) Northern blot of 5 μg of total RNA extracted from tumor (T) and normal (N) biopsy specimens of the oral cavity. (B) Northern blot of 2 μg of poly(A)$^+$ RNA extracted from multiple human organs. Northern blots were probed with a *headpin* specific cDNA probe as indicated in Materials and Methods.

FIG. 6. RH mapping of human *headpin*. *Headpin* is located between WI-4461 and CHLC.GATA2E06 which locates at 18q21.3.

visible in the lane containing normal mucosa, but no signal was present from the tumor sample (Fig. 4A). This transcript size is comparable to the 3.0-kb mRNA species encoding *maspin*, but is longer than the 1.7 kb mRNA reported for SCCA1 (8, 14). The 12-lane MTN blot demonstrated no detectable hybridization signals from any of the different normal tissue types (Fig. 4B). *Headpin* transcript was also detected in normal skin by RT-PCR (data not shown).

Differential tissue expression of headpin. Differential expression of *headpin* was demonstrated by relative RT-PCR. Once *headpin* had been cloned and sequenced, *headpin* specific primers were chosen that would specifically amplify the *headpin* message from reverse transcribed total RNA obtained from the biopsy normal and tumor specimens. The results of relative RT-PCR data demonstrating the underexpression of *headpin* in tumor samples compared to their normal counterparts are seen in Fig. 5. Reverse transcription reactions of the total mRNA from the tissue samples were run in duplicate. The expression pattern illustrates clearly that patient tumor biopsy samples, T1, T2, T3, and T4 produce the *headpin* message in substantially lower amounts than their normal counterparts. The mechanism for the loss of *headpin* in squamous carcinoma may be mutation, deletion or downregulation, but is presently unknown. Additionally, we examined 7 established head and neck tumor cell lines and found *headpin* expressed in significantly lower amounts compared to the normal biopsy specimens based on the addition of equal amounts of cDNA in the RT-PCR mixture (data not shown). Currently, the consequences of *headpin* downregulation in squamous cell carcinoma of the head and neck is not known. However, one possibility is that *headpin* expression could be disadvantageous to tumor invasion mechanisms that rely on proteolytic degradation of extracellular matrix proteins.

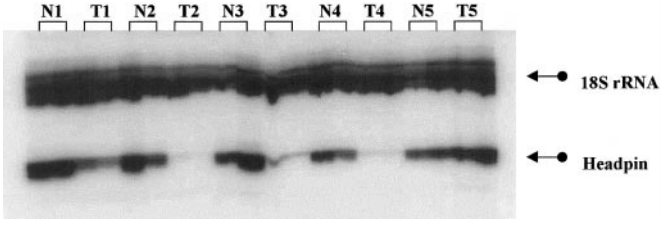

FIG. 5. Relative RT-PCR of *headpin* transcript in normal and tumor site-matched biopsy specimens. The positions of headpin and the control (18S RNA) are indicated by arrows. Normal mucosal biopsy specimen samples are indicated by "N" and tumor biopsy samples by "T."

Localization of headpin to chromosome 18q. We utilized radiation hybrid mapping to determine the chromosomal location of *headpin*. The results of the mapping study place *headpin* between the markers WI-4461 and CHLC.GATA2E06 which locates in the area of 18q21.3/18q22 (Fig. 6). This establishes *headpin* among the other ov-serpins such as *SCCA1, SCCA2,* plasminogen activator inhibitor-2 (*PAI-2*) and *maspin* (4). This area of chromosome 18 is actively studied because of the known breakpoints and LOH that occur on 18q in head and neck cancer and others (6, 9, 15–17). Already, four members of the ov-serpin family have been mapped to a 600-kb region within 18q21.3/18q22; *cen*-maspin, *SCCA2, SCCA1, PAI-2-tel* (4). *Maspin*, a class II tumor suppressor gene, is downregulated in mammary epithelial carcinoma (18). The loss of expression of *maspin* DNA is a consequence of regulatory changes rather than genomic changes (19). *Headpin* may represent an equivalent gene to *maspin* in head and neck cancer.

Headpin is a differentially expressed, novel serine proteinase inhibitor that belongs to the ov-serpin family and demonstrates a hinge region consensus sequence that predicts an inhibitory function. *Headpin* was cloned from a keratinocyte cDNA library and its expression pattern by Northern blot analysis indicates that it is most likely produced by keratinizing epithelium. The endogenous expression of *headpin* in normal oral keratinocytes and its absence or downregulation in squamous cell carcinoma of the oral cavity raises the question of whether

headpin may be a marker for squamous differentiation or a gene disadvantageous to tumor function. *Headpin* has been grouped into the cluster of serpins located at chromosome 18q21.3/18q22. This region is a known area for LOH and other deletional events often associated with head and neck cancer.

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