BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 580–586 (1998)
ARTICLE NO. RC988793

Structural Characterization and Mapping of the Normal Epithelial Cell-Specific 1 Gene

Liuying Luo,*,† Jo-Anne Herbrick,‡ Stephen W. Scherer,‡,§ Barbara Beatty,†,¶ Jeremy Squire,†,¶ and Eleftherios P. Diamandis*,†,[1]

**Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada; †Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada; ‡Department of Genetics, Hospital for Sick Children, Toronto, Ontario, Canada; §Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada; and ¶Ontario Cancer Institute/Princess Margaret Hospital, Toronto, Ontario, Canada*

Received May 12, 1998

The normal epithelial cell-specific 1 (NES1) gene is a recently identified novel serine protease-like gene which is down-regulated during breast cancer progression. The gene product has 34–42% identity with the members of three distinct serine protease families: the trypsin-like family, activators of kringle domain-containing growth factors, and the kallikrein family (X. L. Liu et al., (1996) *Cancer Res* 56, 3371–3379). Although the cDNA of this gene has been cloned, its genomic structure and chromosomal position are not as yet known. Here, we report the genomic characterization and mapping of the NES1 gene. By subcloning and sequencing a PAC clone containing the complete NES1 gene, we were able to characterize the structure of this gene. The NES1 gene spans 5.5 kb and is composed of five coding exons and one untranslated exon. The positions of the introns were similar to trypsinogen, prostate specific antigen (PSA), and tissue plasminogen activator (TPA). NES1 gene was also localized with somatic cell mapping, radiation hybrid mapping, and fluorescence *in situ* hybridization techniques to chromosome 19q13.3–q13.4, the same region where the human kallikrein gene family resides. Taken together, our results suggest that the NES1 gene originates from the same ancestor as trpsinogen, PSA, and TPA, but remains in close proximity to PSA.

Key Words: **breast cancer; gene mapping; kallikrein genes; NES1 gene; NES1 genomic structure.**

The normal epithelial cell-specific 1 (NES1) gene is a recently identified serine protease which appears to be down regulated during breast cancer progression. Its cDNA sequence has been determined and the gene has been predicted to encode an 276 amino acid protein of approximately 30 kDa molecular mass. The physiological function of the protein is unknown. However, it is recognized as a serine protease because the aminoacid residues known to be crucial for substrate binding, specificity and catalysis of the serine proteases, including serine, histidine and aspartic acid are conserved in the predicted NES1 protein. By homology comparison it has 34-42% identity and 50-63% similarity with members of three distinct families of serine proteases, namely the trypsin family (such as human pancreatic trypsinogen III), activators of kringle domain-containing growth factors (such as human tissue plasminogen activator; TPA) and the kallikrein family (1).

Trypsin, a digestive enzyme, is produced as its inactive form trypsinogen in pancreatic acinar cells, then secreted into the gut and activated. The trypsin gene family has at least 10 members in humans (2). The genes have been localized to chromosome 7 (3). TPA is a member of the kringle domain-containing growth factors. It converts the proenzyme plasminogen to plasmin, which degrades blood clots (4). This gene is on chromosome 8 (5). The human kallikrein gene family has three members, including the pancreatic/renal kallikrein (KLK1), the glandular kallikrein (KLK2) and prostate specific antigen (PSA, KLK3) (6). These three genes have been all cloned and well characterised (7-

[1] Correspondence and reprints: Dr. E. P. Diamandis, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, M5G 1X5, Canada. E-mail: ediamandis@mtsinai.on.ca.

Nonstandard abbreviations: NES1, normal epithelial cell-specific 1 gene; PSA, prostate specific antigen; TPA, tissue plasminogen activator; KLK, kallikrein; Kb, kilobase; PAC, P-1 derived artificial chromosome; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; SSC, saline-sodium citrate; FITC, fluorescein isothiocyanate; cR, centiray; kDa, kilodalton; bp, base pairs.

0006-291X/98 $25.00

9). The distinct feature of this gene family is that all the members cluster on a single locus on chromosome 19q13.3–q13.4 (10).

PSA is a well-recognized marker for prostate cancer. In males, it is predominantly produced in prostatic epithelial cells and secreted into the seminal plasma, where it serves to degrade the gel-like seminogelin proteins to increase sperm motility (11,12). In females, it has been shown that breast has the ability to produce PSA and the production is down regulated in malignant breast tumors (13,14).

There are several interesting similarities between NES1 and PSA: (a) Both have sequence similarity as previously described (1); (b) The NES1 gene encodes a serine protease which is predicted to be secreted, as is the case with PSA; (c) NES1 is down-regulated during breast cancer progression, a situation that strikingly resembles PSA gene expression in breast cancer. These common features between PSA and NES1 led us to isolate the entire NES1 gene, determine its chromosomal localization and genomic structure and compare it to the PSA gene. Our findings may contribute to a better understanding of the relationship between NES1 and other members of the serine protease gene superfamily, especially PSA, and thus provide clues for NES1 physiological function and regulation.

MATERIALS AND METHODS

Amplification of NES1 gene by polymerase chain reaction (PCR). Two pairs of NES1 gene-specific primers were designed based on the published NES1 cDNA sequence (1) (Genbank accession number AF024605). They were 4S, 5′-GCTTCCCTACCGCTGTGCT-3′, 4AS, 5′-CACTCTGGCAAGGGTCCTG-3′; 3S, 5′-GATCACCTGCTGCTTCTTC-3′ and 3AS, 5′-GCACTGGTCTCCGGGCTGA-3′. PCR was performed with both human genomic DNA from normal individuals and plasmid containing full length NES1 cDNA (kindly provided by Dr. V. Band) (1). PCR was carried out in a 20 microliter reaction mixture, containing 100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (deoxynucleoside triphosphates), 150 ng primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Diagnostic Systems Inc, Branchburg, NJ 08876, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94 °C for 12 minutes to activate the Taq Gold DNA polymerase, followed by 30 cycles of 94 °C for 30 s, 62 °C for 1 min and a final extension at 62 °C for 10 min. The PCR products were electrophoresed on 2.5% agarose gels.

Cloning and sequencing to verify the identity of the PCR products. The PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, 92008) following the manufacturer's instructions. The inserts were sequenced with vector-specific primers from both directions using an automated DNA sequencer.

Chromosomal localization of the NES1 gene by somatic cell hybrid and radiation hybrid mapping. The mapping was performed by PCR with NES1 gene specific primer pairs 4S/4AS and 3S/3AS. A panel of human-rodent somatic cell hybrids containing single human chromosomes (NIGMS Human/Rodent, somatic cell hybrid, mapping panel #2) and a whole-genome radiation hybrid panel (Genebridge4, Research Genetics, Huntsville, AL) were screened. PCR was carried out as described above but 25 ng of DNA was used per reaction. The results of PCR using the radiation hybrid panel were submitted to the Whitehead Institute/MIT Centre for Genome Research (http://www.genome.wi.mit.edu) for statistical evaluation.

Screening a human genomic DNA library. A P-1 derived artificial chromosome (PAC) library (15) spotted on nylon membranes was screened by hybridization. The PCR product generated with the described primers 4S/4AS was purified, labeled with ^{32}P by the random primer method and used as a probe. The filters were hybridized in 15% formamide, 500 mM Na_2HPO_4, 7% SDS, 1% BSA (w/v) at 65 °C overnight, then washed sequentially with 2× SSC, 1× SSC, 0.2× SSC containing 0.1% SDS at 65 °C and then exposed to X-ray film.

Mapping of NES1 with fluorescence in situ hybridization. The isolated PAC clone containing the NES1 gene (PAC 43B1) was used as a probe. The regional assignment of the PAC clone was determined by fluorescence in-situ hybridization (FISH) to normal human lymphocyte chromosomes counterstained with propidium iodide and 4′, 6-diamidin-2-phenylindol-dihydrochloride (DAPI) following published methods (16,17). Biotinylated probe was prepared by nick translation and detected with avidin-fluorescein isothiocyanate (FITC), followed by biotinylated anti-avidin antibody and avidin-FITC. Images of metaphase preparations were captured by a thermoelectrically cooled charge coupled camera (Photometrics, Tucson, AZ). Separate images of DAPI banded chromosomes (16) and of FITC targeted chromosomes were obtained, pseudo colored blue (DAPI) and yellow (FITC) and merged electronically using Adobe Photoshop 3.0 software. The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern generated by the DAPI counterstained image (16,18,19).

Subcloning and sequencing. The PAC clone containing the NES1 gene (~150 Kb in length) was digested with restriction enzymes *Xba*I and *Not*I. The digested PAC was run on an agarose gel and a 15 Kb fragment was identified to contain the NES1 gene by Southern blot hybridization. This fragment was subcloned into the plasmid vector pZERO™-II using procedures recommended by the manufacturer (Invitrogen, Carlsbad, CA). This plasmid was then subjected to sequencing using automated procedures along with NES1 cDNA specific and walking sequencing primers.

RESULTS

Amplification of the NES1 Gene by PCR

We designed two sets of PCR primers 3S/3AS and 4S/4AS based on the published cDNA sequence, then amplified either human genomic DNA or NES1 cDNA cloned into a plasmid vector. Theoretically, the sizes of the PCR products amplified with primer pairs 3S/3AS and 4S/4AS are 213 bp and 212 bp, respectively based on the cDNA sequence. With plasmid DNA as a target, the obtained PCR products have similar sizes. However, when we amplified human genomic DNA, primer pair 3S/3AS generated the expected size product while primer pair 4S/4AS generated a product of about 500 bp (Figure 1), suggesting that primer pair 4S/4AS was amplifying parts of two exons along with the sequence of an intervening intron. This was verified as described below.

Cloning and Sequencing of the PCR Products

In order to verify the identity of the PCR products with primer pairs 3S/3AS and 4S/4AS from human genomic DNA, we cloned them into the pCRII-TOPO vector and the inserts were sequenced. The sequence of

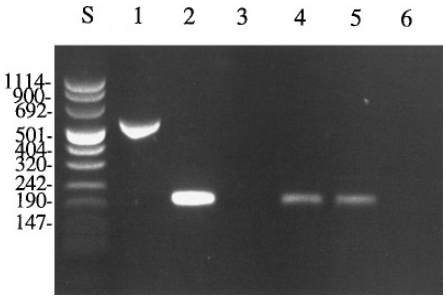

FIG. 1. Amplification of the NES1 gene by PCR. Lane S shows the molecular weight standards with lengths in base pairs as shown on the side; lane 1, the human genomic DNA amplified with primer pair 4S/4AS; lane 2, the NES1 cDNA amplified with primer pair 4S/4AS; lane 3, the negative control; lane 4, the human genomic DNA amplified with primer pair 3S/3AS; lane 5, NES1 cDNA amplified with primer pair 3S/3AS; and lane 6, the negative control.

the PCR product generated by primers 3S/3AS was found to be identical to the corresponding sequence of the NES1 gene cDNA. The sequence of the PCR product generated by primers 4S/4AS was also identical to the corresponding sequence of the NES1 gene cDNA but there was an additional 331 bp intervening sequence, representing an intron (intron IV, see below).

Mapping of the NES1 Gene

We used the PCR procedures mentioned above to map the NES1 gene, using somatic cell hybrid and radiation hybrid mapping. We examined a panel of somatic cell hybrids each containing a single human chromosome with primer pairs 4S/4AS and 3S/3AS. This experiment showed that the NES1 gene resided on chromosome 19 (Figure 2). A similar PCR analysis was performed on a panel of Genebridge4 radiation hybrid DNA. The expected PCR product was amplified from 14 out of 93 DNAs from the hybrid cell lines (data not shown). Computer analysis of these data localized the NES1 gene on chromosome 19, between markers NIB1805 and WI-5264. The distance between NES1 and NIB1805 was 4.08 cR. Marker NIB1805 (Genbank ID T16756) resides 347.13 cR from the top of chromosome 19. In addition, the PSA gene (WI-9055; Genbank ID M21896) has been previously mapped with the same method to 351.84 cR from the top of chromosome 19. These data allowed us to construct a tentative physical map of the region containing the NES1 and PSA genes (Figure 3).

Screening a Human Genomic DNA Library

Screening of the human PAC genomic library (15) yielded a positive clone containing the NES1 gene (PAC 43B1). PCR analysis with PSA-specific primers further indicated that the PSA gene was not present on this PAC clone (data not shown).

Fluorescence in Situ Hybridization

The PAC clone was mapped by fluorescence in-situ hybridization (Figure 4). By this technique, NES1 was mapped to chromosome 19q13.3–q13.4, in full agreement with the somatic cell hybrid and radiation hybrid mapping.

Genomic Structure of the NES1 Gene

The PAC clone 43B1 was subcloned into the plasmid vector pZERO-II and 6.5 kb of the insert was sequenced. By comparison with the published cDNA sequence, we found that the genomic DNA sequence contains the entire NES1 gene. Intron-exon boundaries were determined by aligning the genomic sequence with the cDNA. Figure 5 shows the genomic organization of the NES1 gene. This sequence has been deposited in Genbank (accession number: AF055481). The NES1 gene is composed of one untranslated exon and five coding exons. All exon-intron junctions followed the GT-AG rule (20), except for the donor site of intron IV, which started with GC. Although the exact transcription initiation site is not known, according to the available cDNA sequence and the size of the NES1 mRNA (1), a TATA box variant TTAAAA was found 35 bp upstream of the cDNA start site.

DISCUSSION

The DNA sequence presented in figure 5 shows that the NES1 gene is made up of 6 exons separated by 5 introns and spans 5.5kb. All splice junctions well agree with the consensus sequence GT-AG, except the splice donor of intron IV, which has GC instead of GT. This

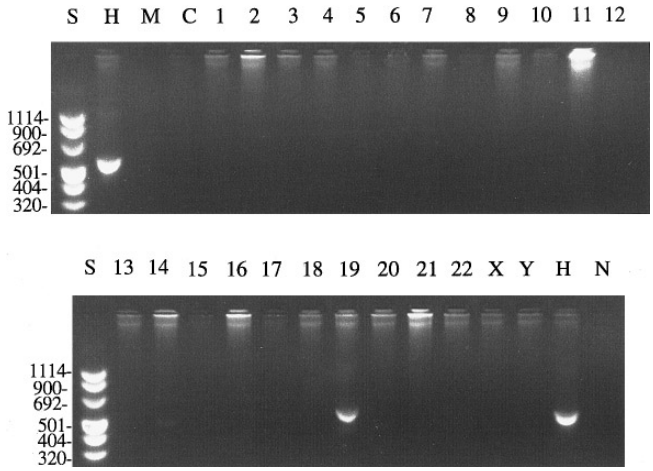

FIG. 2. Chromosomal localization of the NES1 gene obtained by PCR analysis of somatic cell hybrid DNA. Lanes are labeled with numbers 1–22, and X and Y represent human chromosomes retained in each hybrid. DNA controls are from human (H), Chinese hamster (C), and mouse (M); negative control (N) and size markers (S) are indicated.

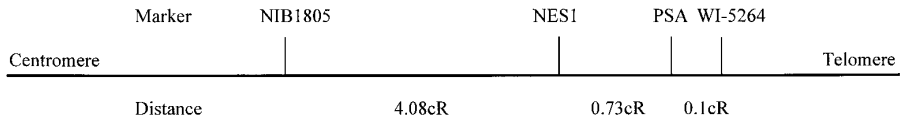

FIG. 3. Schematic diagram showing a region of chromosome 19q13.3–q13.4 and the position of the NES1 gene relative to other known markers and the PSA gene. The distances in cR are not drawn to scale.

rare variant has also been observed in a few other genes (21-23). A TA stretch of TTAAAA was found 35 bp upstream from the available cDNA sequence, suggesting that it is likely the variant of the TATA box. This variant is also found in other genes (24). Furthermore, there is no TATA like sequence further upstream. Usually, the most frequent mRNA start site exists 22-29 bp downstream from the TATA box (24). Indeed, the sequence GCAGAG, just 26 bp downstream from this putative TATA box (Figure 5) closely resembles the consensus mRNA cap site (T/GCAGTC) (24). This sequence, GCAGAG, is also found in another serine protease gene, bovine prothrombin, and it has been shown that the mRNA starts from the first nucleotide G (23). Whether this is the genuine mRNA start site of the NES1 gene remains to be experimentally determined.

Since the NES1 gene has sequence homology with trypsinogen, PSA, and TPA, the genomic organizations of these genes were compared. There are three distinct features in secreted serine proteases: signal peptide, cleavage site, and catalytic triad. The catalytic triad serine[229], histidine[86] and aspartic acid[137] (the residue numbers shown are based on the NES1 gene) are crucial residues for substrate binding, specificity, and catalysis of serine protease(25). The cleavage site is the position where the proenzyme is cleaved. The signal peptide is the sequence that is recognized by the proteins involved in the secretory pathway. In the NES1 gene, the cleavage site is predicted to be at residue 42 and there is a putative signal peptide in the NH_2-terminus of the NES1 according to the hydropathicity study (1). The exon-intron organization of serine protease genes has been well documented. Irwin et al have proposed that the serine protease genes can be grouped into five different types based on the intron position (23). Trypsinogen, PSA and TPA all belong to the group that has the following characteristics: (a). The region encoding the signal peptide is in the first coding exon; (b). The catalytic residues histidine and serine are separated in different exons and almost immediately adjacent to their exon boundaries. There is only one intron

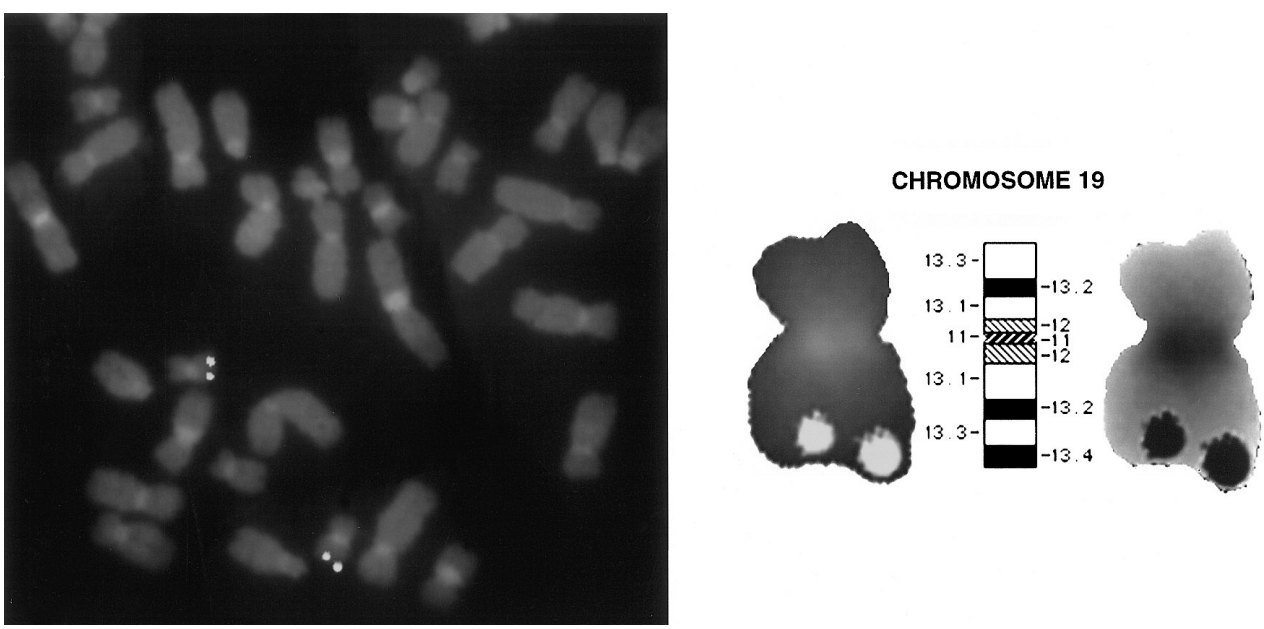

FIG. 4. Regional mapping of the NES1 gene by fluorescence in situ hybridization to normal human lymphocyte chromosomes counterstained with DAPI. Biotinylated P-1 artificial chromosome (PAC) genomic NES1 probe was detected with avidin-fluorescein isothiocyanate (FITC). Separate images of DAPI counterstained metaphase chromosomes and of NES1 probe hybridization signals were captured and overlaid electronically as described in the Methods section. Part of a representative metaphase preparation is shown to indicate the position of the NES1 probe FISH signals that are visible as yellow fluorescent signals on the long arm of chromosome 19. A DAPI-banded chromosome 19 together with a schematic ideogram is shown to indicate that the NES1 probe hybridizes to band q13.3–q13.4.

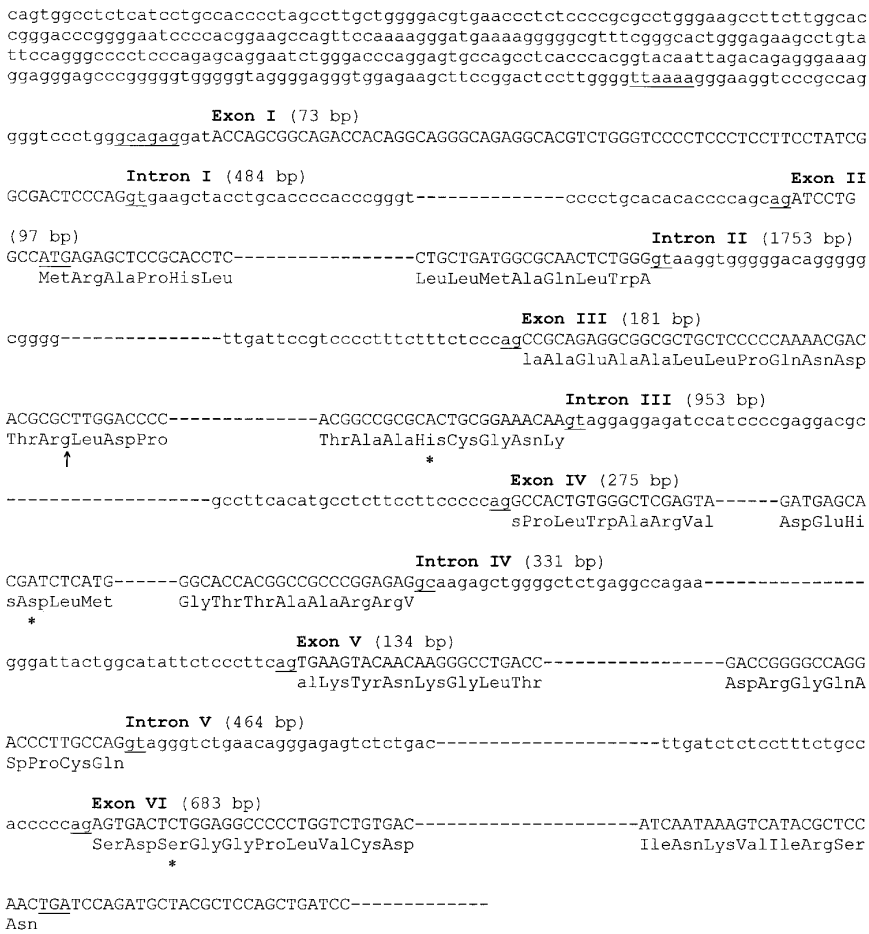

FIG. 5. Genomic organization and partial nucleotide sequence of the NES1 gene. Sequence gaps can be found in Genbank accession #AF055481. Lower case letters represent introns, the 5′ untranslated region, and the promoter sequence. Upper case letters represent exons, and the translated aminoacids are shown underneath. The underlined sequence indicates the putative TATA box and mRNA cap site, intron–exon junctions, translation start codon, and stop codon. The asterisks indicate the catalytic residues. The arrow shows the cleavage site.

between the exons coding for the histidine and aspartic acid residues. (c). The cleavage site is localized in the same exon as the histidine residue. When the genomic organization of the NES1 gene was analyzed, it shared the same intron-exon organization as trypsin, PSA, and tissue plasminogen activator (Figure 5 and Figure 6), suggesting that these serine protease genes have a common origin. It has been reported that in spite of the limited sequence similarity between different serine proteases which may share a common ancestor, they have high degree of homology in protein conformation as determined by x-ray crystallography (26). This phenomenon has also been observed in the sequence and structural studies of trypsinogen and PSA (27-29). Therefore, we speculate that NES1 may have a similar protein conformation and enzymatic action as PSA and trypsinogen.

The human kallikrein gene family is known to be clustered in a 60 kb region on chromosome 19q13.3-q13.4 in the order of: centromere-KLK1-PSA-KLK2-telomere. The distance from PSA to KLK1 and to KLK2 are 31 kb and 12 kb, respectively (10). In our study, the NES1 gene was mapped to chromosome 19q13.3–q13.4, in the same region as the human kallikrein gene family. However, other related serine proteases reside on different chromosomes, e.g. trypsin (chromosome 7) (3) and tissue plasminogen activator (chromosome 8) (5). Radiation hybrid mapping placed the NES1 gene between markers NIB1805 and WI-5264. The latter is only 0.1 cR away from the PSA gene. These data indicate that the NES1 gene is only about 0.73 cR centromeric to the PSA gene and that the arrangement of the genes at this locus is: 19centromere-NES1-KLK1-PSA-KLK2-19telomere. The fluorescence in-situ hybridization data fully support the localization of the NES1 gene in this region.

Taken together, our data indicate that NES1 shares a similar genomic organization with PSA, trypsinogen

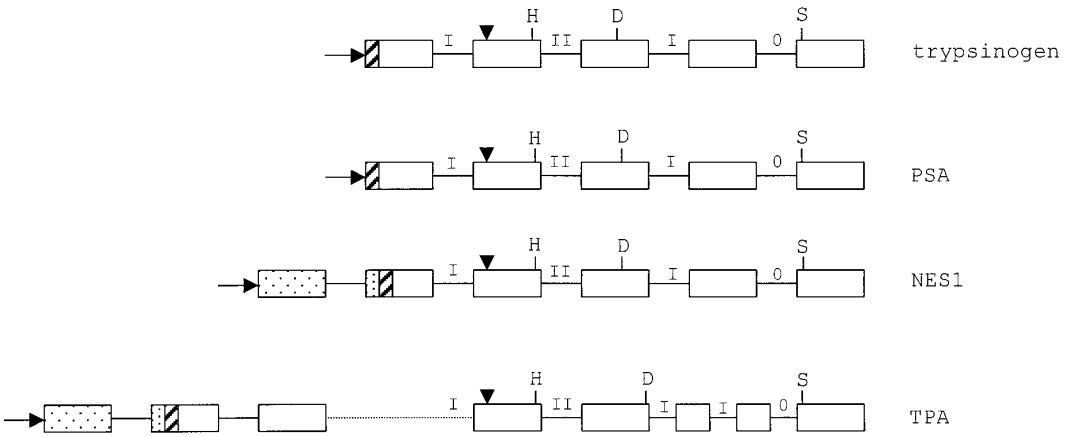

FIG. 6. Schematic diagram showing the comparison of genomic exon–intron organization of trypsinogen, PSA, NES1, and TPA. Exons are shown by open boxes and introns are shown by connecting lines. The dotted boxes represent untranslated exons and the shaded boxes indicate the regions that encode the signal peptides. The arrow heads and vertical lines on top of the boxes indicate the relative positions of the cleavage sites and the catalytic triad inside the exons, respectively. H denotes histidine; D, aspartic acid; and S, serine. The arrows on the left represent the promoter region. The dotted line inside the TPA gene denotes the exons that encode the nonprotease domain. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon: I denotes that the intron occurs after the first nucleotide of the codon; II, the intron occurs after the second nucleotide of the codon; 0, the intron occurs between codons. The boxes and line lengths are not drawn to scale. Figure was adapted and modified from Ref. (23).

and TPA, and resides in close proximity to PSA. It is likely that these serine proteases are all derived from a common ancestral gene. During evolution, the members of this ancestral gene family have been split to different chromosomes, but NES1 and PSA remains close to each other. Interestingly, another gene, named Protease M, has been reported to be a serine protease and has 39 % protein sequence identity to PSA. It is localized on chromosome 19q13.3-q13.4 and its expression is also reduced in breast cancer (30). NES1 gene and protease M gene only have 47 % DNA sequence identity (data not shown), making it unlikely that they belong to the same gene family.

How and why these serine proteases are down regulated in breast cancer remain unknown. The characterization of the full genomic structure and localization of the NES1 gene will help us understand better its function and relationship with other genes and may ultimately contribute to the understanding of the involvement of the serine protease genes in breast cancer initiation and progression.

ACKNOWLEDGMENTS

We thank Dr. V. Band for kindly providing a plasmid containing the cDNA of the NES1 gene and Zong Mei Zhang for technical assistance. This work was partially funded by the Canadian Breast Cancer Research Initiative of the National Cancer Institute of Canada (to E. P. Diamandis), the Canadian Genome Analysis and Technology Program (CGAT) (to J. Squire), and the Medical Research Council of Canada, Genome Resource Facility (to S. W. Scherer).

REFERENCES

1. Liu, X. L., Wazer, D. E., Watanabe, K., and Band, V. (1996) *Cancer Res.* **56,** 3371–3379.
2. Emi, M., Nakaura, Y., Ogawa, M., Yamamoto, T., Nishide, T., Mori, T., and Matsubara, K. (1986) *Gene* **41**(2–3), 305–310.
3. Honey, N. K., Sakaguchi, A. Y., Quinto, C., Macdonal, R. J., Bell, G. I., Craik, C., and Naylor, S. L. (1984) *Somat. Cell Mol. Genet.* **10**(4), 369–376.
4. Collen, D. (1980) *Thromb. Haemostasis* **43,** 77–89.
5. Yang-Feng, T. L., Opdenakker, G., Volckaert, G., and Francke, U. (1986) *Am. J. Hum. Genet.* **39**(1), 79–87.
6. McCormack, R. T., Rittenhouse, H. G., Finlay, J. A., Sokoloff, R. L., Wang, T. J., Wolfert, R. L., Lilja, H., and Oesterling, J. E. (1995) *Urology* **45**(5), 729–744.
7. Evans, B. A., Yun, Z. X., Close, J. A., Tregear, G. W., Kitamura, N., Nakanishi, S., Callen, D. F., Baker, E., Hyland, V. J., Sutherland, G. R., and et al. (1988) *Biochemistry* **27**(9), 3124–3129.
8. Schedlich, L. J., Bennetts, B. H., and Morris, B. J. (1987) *DNA* **6**(5), 429–437.
9. Riegman, P. H., Vlietstra, R. J., van der Korput, J. A., Romijn, J. C., and Trapman, J. (1989) *Biochem. Biophys. Res. Commun.* **159**(1), 95–102.
10. Riegman, P. H., Vlietstra, R. J., Suurmeijer, L., Cleutjens, C. B., and Trapman, J. (1992) *Genomics* **14**(1), 6–11.
11. Lilja, H. (1985) *J. Clin. Invest.* **76,** 1899–1903.
12. Lilja, H., Oldbring, J., and Rannevik, G. (1987) *J. Clin. Invest.* **80,** 281–285.
13. Yu, H., Giai, M., Diamandis, E. P., Katsaros, D., Sutherland, D. J., Levesque, M. A., Roagna, R., Ponzone, R., and Sismondi, P. (1995) *Cancer Res.* **55**(10), 2104–2110.
14. Diamandis, E. P., and Yu, H. (1995) *J. Clin. Endocrinol. Metab.* **80,** 1515–1517.
15. Ioannou, P. A., Amemiya, C. T., Garnes, J., Kroisel, P. M., Shizuya, H., Chen, C., Batzer, M. A., and de Jong, P. J. (1994) *Nat. Genet.* **6**(1), 84–89.
16. Heng, H. H., and Tsui, L. C. (1993) *Chromosoma* **102**(5), 325–332.
17. Lichter, P., Tang, C. J., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990) *Science* **247,** 64–69.
18. ISCN. (1978) *Cytogenet. Cell. Genet.* **21,** 309–404.

19. Francke, U. (1994) *Cytogenet. Cell. Genet.* **65**(3), 206–218.
20. Mount, S. M. (1982) *Nucl. Acids Res.* **10,** 459–472.
21. Dush, M. K., Sikela, J. M., Khan, S. A., Tischfield, J. A., and Stambrook, P. J. (1985) *Proc. Nat. Acad. Sci. U. S. A.* **82,** 2731–2735.
22. Wieringa, B., Hofer, E., and Weissmann, C. (1984) *Cell* **37,** 915–925.
23. Irwin, D. W., Robertson, K. A., and MacGillivray, T. A. (1988) *J. Mol. Biol.* **200,** 31–45.
24. Bucher, P. (1990) *J. Mol. Biol.* **212,** 563–578.
25. Schultz, R. M., and Liebman, M. N. (1992) *in* Textbook of Biochemistry, 3rd ed. (Devlin, T. M., Ed.), pp. 102–112, Wiley–Liss, New York.
26. Delbaere, L. T. J., Hutcheon, W. L. B., James, M. N. G., and Thiessen, W. E. (1975) *Nature* **257,** 758–763.
27. Bode, W., Chen, Z., Bartels, K., Kutzbach, C., Schmidt-kastner, G., and Bartunik, H. (1983) *J. Mol. Biol.* **164,** 237–282.
28. Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W. (1974) *J. Mol. Biol.* **89,** 73–101.
29. Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1974) *J. Mol. Biol.* **83,** 185–208.
30. Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S. C., and Sager, R. (1996) *Mol. Med.* **2**(5), 624–636.