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Seminoma-derived Nagao isozyme is encoded by a germ-cell alkaline phosphatase gene

(gene structure/tissue specificity/tumor markers/synthetic peptides/three-dimensional modeling)

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ABSTRACT **A full-length placental alkaline phosphatase (PLAP) cDNA was used to identify and clone the PLAP-like Nagao isozyme gene from human genomic libraries. The entire nucleotide sequence of the gene reveals the existence of 11 exons interrupted by 10 small introns (76–427 base pairs). Putative regulatory sequences have been identified in the promoter regions as well as dispersed in the introns. The deduced amino acid sequence of the Nagao isozyme indicates that the mature molecule is composed of 513 amino acids, of which 12 residues are different from the sequence of PLAP (98% homology). A sequence derived from exon III of the Nagao isozyme gene was used to synthesize a peptide (NH_2-Lys-Leu-Gly-Pro-Glu-Thr-Phe-Leu-Ala-COOH) that contains two mutations with respect to the corresponding PLAP sequence. This peptide elicited rabbit polyclonal antibodies that reacted specifically with the seminoma Nagao isozyme but not with PLAP in electrophoretic transfer blots. These results indicate that the tumor, and possibly the normal testis, Nagao isozyme is encoded by a gene referred to as germ-cell alkaline phosphatase gene that differs from the PLAP gene expressed by syncitiotrophoblastic cells.**

Human alkaline phosphatases (APs) are encoded by a multilocus enzyme system (1). The recent cloning of cDNAs coding for placental alkaline phosphatase (PLAP; EC 3.1.3.1) (2–4), tissue-unspecific or liver/bone/kidney-type AP (5), and intestinal AP (6, 7) confirms the existence of at least three distinct genes in this family.

PLAP is of particular interest, recently reviewed in ref. 8. PLAP expression is usually confined to the syncitiotrophoblast from 12 weeks of pregnancy to term (9). PLAP shows the highest degree of allelic variation of any human enzyme studied to date, displaying a polymorphism evidenced by electrophoretic and immunochemical techniques (10–12). PLAP was found to be reexpressed in a patient with terminal bronchogenic cancer ("Regan isozyme") by Fishman *et al.* (13). A PLAP-like isozyme ("Nagao isozyme") was soon after discovered by Nakayama *et al.* (14) that differed from PLAP by its slower migration on starch gel and its greater sensitivity to the uncompetitive inhibitor L-leucine. The Nagao isozyme is normally expressed in trace amounts in testis (15) and thymus (16). Enhanced expression of Nagao isozyme is found in germ-cell tumors of the testis, particularly seminoma (17), where it serves as a useful marker of the disease in these patients (18). The question of identity between PLAP and Nagao isozyme has remained unresolved. Studies using allotype-specific polyclonal (19) and monoclonal antibodies (18, 20) against PLAP allelic variants allowed the detection of structural differences in the Nagao isozyme that led to the proposal that a distinct gene locus is responsible for the expression of this enzyme (19–21).

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Abbreviations: PLAP, placental alkaline phosphatase; AP, alkaline phosphatase.

In this paper we report the structure and sequence of the Nagao gene,* here termed "germ-cell AP" gene since this is the lineage where predominant expression is observed. An area of the molecule displaying mutations with respect to PLAP has been exploited to generate anti-peptide polyclonal antisera that react selectively with the seminoma-derived Nagao isozyme.

MATERIALS AND METHODS

Library Screening and Characterization of the Clones. Partial *Mbo* I-digested human placental and spleen DNA genomic libraries constructed in Charon 28 λ phage were kindly provided by Kelly Mayo (Northwestern University, Evanston, IL). The libraries were plated on *Escherichia coli* C600 cells and screened by the method of Benton and Davis (22) using as probe either a 2.0-kilobase (kb) *Eco*RI–*Kpn* I fragment or a 261-base-pair (bp) *Eco*RI–*Bam*HI fragment of the PLAP cDNA (ref. 3; Fig. 1*A*) radiolabeled with [^{32}P]dCTP by using an oligolabeling kit (Pharmacia). Positive clones were plaque purified and expanded as described in Maniatis *et al.* (23), and the DNA was analyzed by restriction enzyme mapping with enzymes purchased from Bethesda Research Laboratories.

Sequence Analysis. Selected genomic fragments were subcloned into M13mp18 and M13mp19 or Bluescript M13 (Stratagene Cloning Systems, San Diego, CA) and sequenced by using the universal 17-mer primer (P-L Biochemicals) and 17- and 18-mer oligonucleotides synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer. Sequencing of the clones was accomplished by the Sanger dideoxy chain-termination procedure (24) using the Klenow fragment of DNA polymerase and dATP[^{35}S] as tracer (25). DNA sequences were assembled, analyzed, and compared by using the Microgenie sequence software (Beckman). Computer modeling of the peptides was performed with an Evans and Sutherland PS300 computer graphics system, using the Discover (version 2.3) software.

Antisera Against a Synthetic Peptide. A 9-mer peptide was synthesized on an Applied Biosystems peptide synthesizer. The peptide was coupled to either keyhole limpet hemocyanin or bovine serum albumin at a ratio of 4:1 (wt/wt) by the glutaraldehyde procedure (26). Coupling of the peptide to CNBr-activated Sepharose 4B was accomplished according to the manufacturer's instructions (Pharmacia) to obtain 10 mg of peptide per ml of Sepharose. A New Zealand White female rabbit was inoculated with 4 mg of keyhole limpet hemocyanin-conjugated peptide emulsified with complete Freund's adjuvant. Equivalent amounts in incomplete Freund's adjuvant were administered at 15-day intervals. Test bleedings were checked for antibody titer in sandwich ELISA with 15 μg of bovine serum albumin-conjugated peptide per ml for coating. Total IgG from positive bleedings

*This sequence is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA) (accession no. J032512).

was isolated on a protein A-Sepharose column (Pharmacia). Subsequently, anti-peptide IgG was bound to the peptide-Sepharose column in 50 mM Tris (pH 8.0) containing 0.15 M NaCl (TBS), eluted with 0.1 M glycine/HCl buffer (pH 2.8), and neutralized with 10× concentrated TBS.

The production of a rabbit antiserum (no. 244) against CNBr fragments of purified PLAP has been published (3).

Tissue Samples. A sample of seminoma was obtained after surgical excision of the tumor from material remaining after pathological examination. This testicular tumor sample contained a Nagao isozyme that displayed enzyme inhibition profiles and monoclonal antibody reactivities characterisitic of the type I PLAP-like enzyme (20). The tissue was homogenized in 50 mM Tris (pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.05% Triton X-100, extracted with 1-butanol, heat-inactivated for 10 min at 65°C, clarified by ultracentrifugation, and applied on a Mono Q FPLC column (Pharmacia). A peak with AP activity eluted at 0.2 M NaCl and was purified further on an H7 monoclonal antibody column as described (27).

A sample of intestinal mucosa was obtained after autopsy and processed similarly except that the heat-inactivation step was omitted and the B10 monoclonal antibody column was used in the last step (27).

RESULTS AND DISCUSSION

Gene Structure and Deduced Protein Sequence. A total of 38 positive clones were isolated after screening the human placental and spleen genomic libraries with the *Eco*RI–*Kpn* I 2.0-kb fragment of the PLAP cDNA. Restriction enzyme analysis and hybridization after Southern blotting indicated that we had cloned two different genes (Fig. 1). The clones λ4 and λ9 were chosen for further studies (Fig. 1). The partial sequence analysis of λ9 indicated that it represented the intestinal AP gene (28) since the amino acid sequence deduced for exons I–III corresponded exactly to the sequence derived from two independently cloned intestinal AP cDNAs (6, 7). The sequence of λ4 (see below) indicates that it encodes a protein different from PLAP, now termed germ-cell AP.

The DNA sequence was obtained for the *Bam*HI, *Pvu* II, and *Sma* I fragments of λ4 found to hybridize with the different subfragments of the PLAP cDNA (Fig. 1). The complete nucleotide sequence of the germ-cell AP gene is presented in Fig. 2. Fig. 3 shows the intron–exon structure and restriction enzyme map of the gene as well as the sequencing strategy. The intron–exon junctions were identified by aligning the genomic sequences with the sequence of the PLAP cDNA. The gene is interrupted by 10 small introns ranging in size from 76 bp (intron IV) to 427 bp (intron V). The exons are small and of comparable size (73–312 bp), with the exception of exon XI (800 bp), which encodes the COOH terminus of the protein and also includes the entire 3′ untranslated region of the cDNA.

The deduced amino acid sequence of the germ-cell AP is presented in Fig. 2. Those amino acids that differ in the PLAP sequence are spelled out immediately underneath the germ-cell AP residues. Major differences between germ-cell AP and PLAP are found in exon I. The 5′ untranslated region immediately upstream of the start codon for translation is different from the corresponding PLAP cDNA sequence (3).

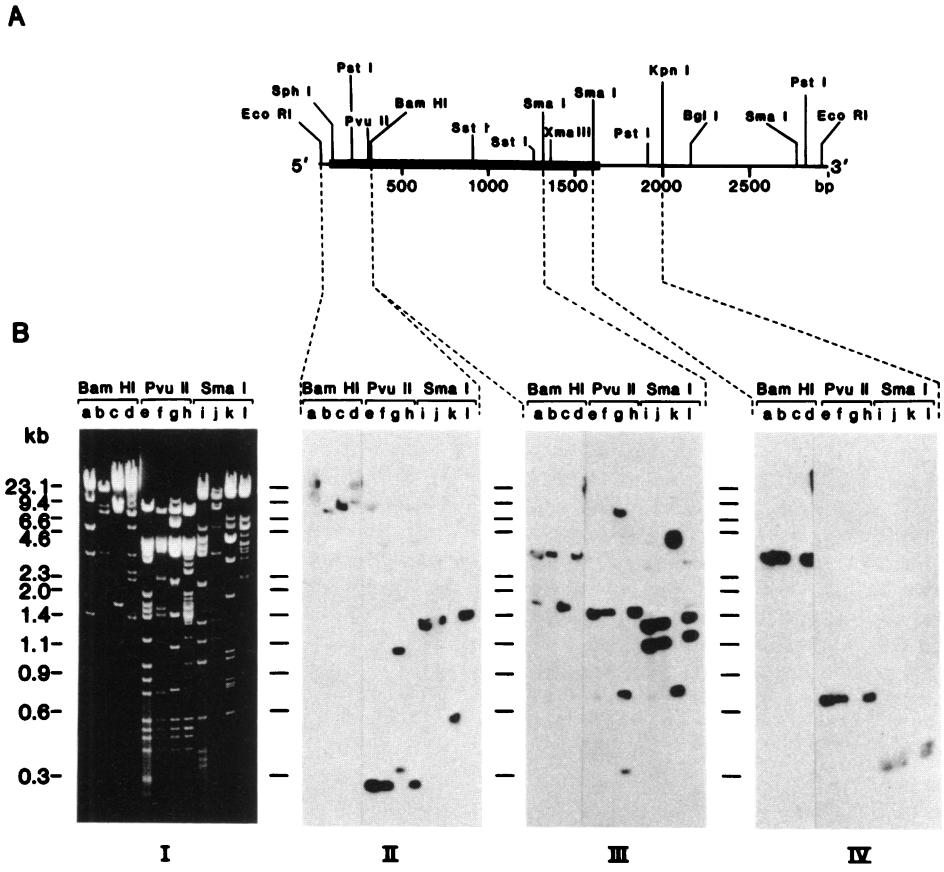

FIG. 1. Restriction enzyme analysis of genomic clones containing PLAP-related sequences. (*A*) Restriction map of the PLAP cDNA (3). (*B*) Southern blot and hybridization analysis of PLAP-related genomic clones. Panel I, restriction enzyme pattern obtained after digestion with *Bam*HI, *Pvu* II, and *Sma* I of genomic clones λ4 (lanes a, e, and i), λ5 (lanes b, f, and j), λ9 (lanes c, g, and k), and λ10 (lanes d, h, and l). Panels II, III, and IV, Southern blots of material in panel I hybridized with radiolabeled 261-bp *Eco*RI–*Bam*HI subfragment, the 989-bp *Bam*HI–*Sma* I subfragment, and the 384-bp *Sma* I–*Kpn* I subfragment of the PLAP cDNA, respectively.

FIG. 2. Nucleotide sequence of the germ-cell AP gene. The consensus sequences for binding of Sp1 transcription factor and "TATA box" are indicated. Arrows indicate a direct repeat. The sequence GGGAGGG repeated 14 times (on either strand) throughout the gene is underlined. The deduced amino acid sequence of the Nagao isozyme is indicated. Those residues that are mutated in the PLAP molecule are spelled out immediately under the substituted position.

The signal peptide sequence of the germ-cell AP is different from the PLAP signal peptide at positions +1, +4, and +5 and shows deletions of three leucines with respect to PLAP (Fig. 2). The mature germ-cell AP protein is composed, as is PLAP, of 513 amino acids. The sequence similarity between these two gene products is 98%. Twelve amino acid mutations are scattered throughout the molecule (Fig. 2). In spite of the high degree of sequence conservation between PLAP and germ-cell AP, the restriction enzyme maps of these two genes are considerably different (Figs. 1*A* and 3).

Putative Regulatory Elements. It is clear that the presence of Nagao isozyme in seminoma represents an enhanced eutopic expression (29) of a gene normally expressed in trace amounts in immature germ cells (15, 16). The possibility that a major gene rearrangement of the PLAP gene would be the basis for the tumor expression of a PLAP-like enzyme has

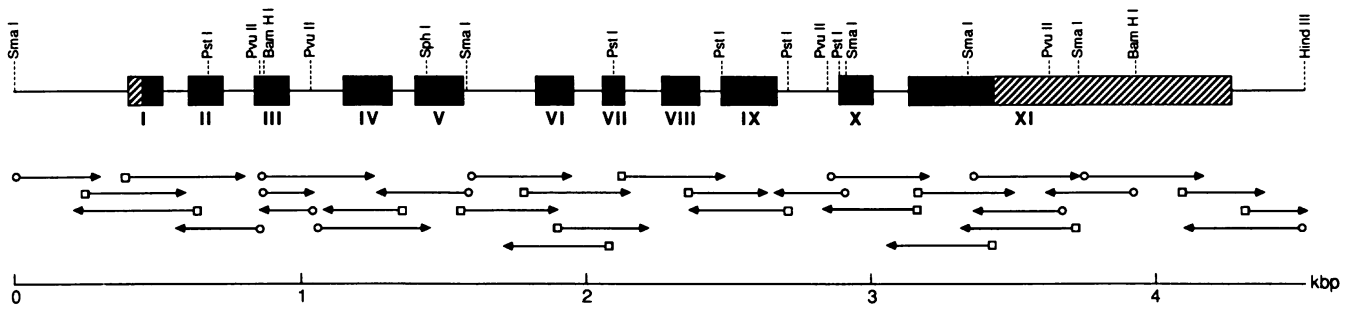

FIG. 3. Restriction enzyme map of the germ-cell AP gene. The gene was sequenced by using universal primers as well as synthetic oligonucleotides. The sequencing strategy is indicated by arrows. Open circles indicate priming by commercially available M13 or Bluescript primers. Open squares indicate priming by oligonucleotides synthesized in our institute. kbp, Kilobase pairs.

recently been ruled out (30). It is of considerable importance to define the mechanisms of regulation as well as trans-activating factors involved in the differential expression of highly homologous genes in syncitiotrophoblastic cells versus germ cells.

The promoter region of the germ-cell AP gene (Fig. 2) contains a TATA box (31) sequence present in its variant form ATTTAAA. This sequence is also found in the promoter of the intestinal AP gene within a conserved region of 25 bp (28). A consensus binding site (TGGGCGGG) (32) for the Sp1 transcription factor is found upstream from the TATA box in the germ-cell AP gene as well as the intestinal AP gene (28). The germ-cell AP gene lacks a consensus CAAT box (33). Several features are unique to the germ-cell AP gene: (*i*) a direct repeat (CCCAG) indicated by arrows in Fig. 2 upstream from the Sp1 binding site; (*ii*) an alternating purine-pyrimidine CA repeated 12 times is located in the first intron (indicated in bold letters in Fig. 2); this direct repeat could form a Z-DNA structure, which may play a role in the regulation of gene expression (34); (*iii*) the sequence GGGGAGGGG or its inverted sequence CCCCTCCCC (both underlined in Fig. 2) is found 14 times throughout the entire length of the gene, primarily in the promoter region and in introns; one such sequence is found in exon VIII and two others are found in the 3′ untranslated region of exon XI.

These unique sequences may be tested in transfection experiments to determine their functional significance.

Generation of Nagao Isozyme-Specific Antiserum. Serological measurements of PLAP and Nagao isozyme levels have proven of clinical value in the management of testicular cancer patients (18, 35). Recent reports have documented the usefulness of these tumor markers also in the evaluation of ovarian cancer patients (36, 37). PLAP and the Nagao isozyme are potentially useful targets for the immunolocalization of primary and metastatic tumors by using radiolabeled monoclonal antibodies (38, 39).

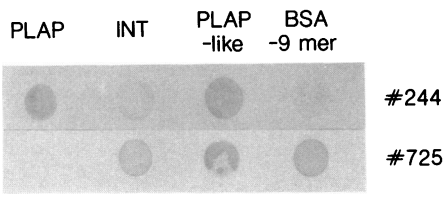

FIG. 4. Reactivities of rabbit antisera produced against CNBr fragments of PLAP (no. 244) and against the synthetic 9-mer peptide (no. 725). Three hundred nanograms of purified PLAP, seminoma Nagao isozyme (PLAP-like), intestinal AP (INT), and bovine serum albumin-9-mer (BSA-9 mer) conjugate was spotted onto nitrocellulose after reduction with 2-mercaptoethanol and boiling in 1% $NaDodSO_4$. The blots were treated as described (3) and allowed to react with purified IgG at a concentration of 100 μg/ml. Detection was accomplished with horseradish peroxidase-labeled secondary antibody.

In this paper, a Nagao isozyme-specific antiserum was generated by injection into rabbits of a synthetic peptide derived from the amino acid sequence deduced from the nucleotide sequence of the germ-cell AP gene. Two amino acid mutations with respect to PLAP are clustered in exon III of the germ-cell AP sequence. This area was chosen to synthesize a peptide in an attempt to elicit polyclonal antiserum with specificity for the tumor enzyme. The synthetic 9-mer NH_2-Lys-Leu-Gly-Pro-Glu-Thr-Phe-Leu-Ala-COOH includes both substitutions with respect to PLAP. As shown in Fig. 4, although the control no. 244 antiserum reacts well with PLAP, germ-cell AP (PLAP-like), and intestinal AP the affinity-purified IgG (no. 725) against the synthetic 9-mer reacts well with the germ-cell AP but does not recognize PLAP. Interestingly, adult intestinal AP is also recognized by this antiserum. The corresponding sequence in exon III of intestinal AP displays the isoleucine → threonine substitution with respect to PLAP but, in this case, the adjacent proline is not substituted (6, 7, 28). Therefore, it seems that threonine

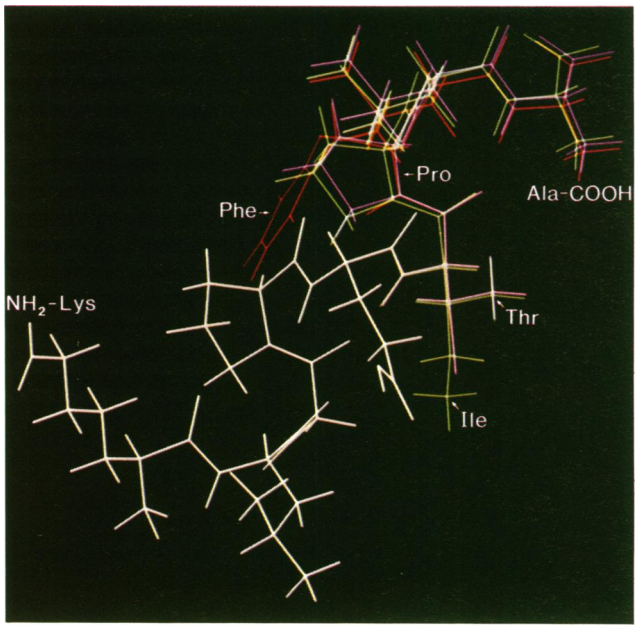

FIG. 5. Computer modeling of the three-dimensional conformation of 9-mer peptides corresponding to the sequences of (*i*) PLAP (NH_2-Lys-Leu-Gly-Pro-Glu-Ile-Pro-Leu-Ala-COOH), in green; (*ii*) germ-cell AP (NH_2-Lys-Leu-Gly-Pro-Glu-Thr-Phe-Leu-Ala-COOH), in red; and (*iii*) intestinal AP (NH_2-Lys-Leu-Gly-Pro-Glu-Thr-Pro-Leu-Ala-COOH), in magenta. This conformation was obtained by adopting a 3-10 helix secondary structure prediction that gives the best fit of the peptide backbones. The relative spatial position of phenylalanine threonine/isoleucine side chains is maintained even when the less than best fit of the α-helix and β-sheet predictions is adopted.

contributes to a greater extent than phenylalanine to the structure of the epitope in this synthetic peptide. These results are more clearly understood by superimposing the structure of the PLAP, germ-cell AP, and intestinal AP 9-mer peptides using a computer graphic modeling software. Fig. 5 shows that the aromatic side chain of the substituted phenylalanine in the germ-cell AP peptide spatially lies far apart from the side chains of threonine. Thus, the three-dimensional conformation renders them unavailable to jointly participate in the structure of the epitope. In terms of antibody recognition, these germ-cell AP and intestinal AP peptide sequences are not distinguishable.

Further work is necessary to synthesize various peptides with the various amino acid substitutions reported in this paper that may represent unique epitopes of the germ-cell AP molecule.

This report demonstrates the existence and reveals the structure of a germ-cell AP gene, different from the PLAP gene, and provides evidence that this gene encodes the Nagao isozyme overproduced by seminoma of the testis.

This paper is respectfully dedicated to Dr. William H. Fishman, whose pioneering discovery of the "Regan isozyme" 20 years ago inspired this field of study in the area of oncodevelopmental biology. We are grateful to Drs. Panagiotis Tsonis, Scott Argraves, and Bob Oshima for their criticisms and suggestions during the course of this work and to Dr. Erkki Ruoslahti for critically reading this manuscript. We thank Ken Dickerson for his help with FPLC purifications, Dr. Michael Pierschbacher for his help with computer modeling, and Mrs. Tami Clevenger for secretarial assistance. This work was entirely supported by Grant CA 42595 from the National Institutes of Health.

1. Stigbrand, T., Millán, J. L. & Fishman, W. H. (1982) *Isozymes* **6**, 93–117.
2. Kam, W., Clauser, E., Kim, Y. S., Kan, Y. W. & Rutter, W. J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8715–8719.
3. Millán, J. L. (1986) *J. Biol. Chem.* **261**, 3112–3115.
4. Henthorn, P. S., Knoll, B. J., Raducha, M., Rothblum, K. N., Slaughter, C., Weiss, M., Lafferty, M. A., Fischer, T. & Harris, H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5597–5601.
5. Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. & Harris, H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7182–7186.
6. Berger, J., Garattini, E., Hua, J. & Undenfriend, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 695–698.
7. Henthorn, P. S., Raducha, M., Edwards, Y. H., Weiss, M. J., Slaughter, C., Lafferty, M. A. & Harris, H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1234–1238.
8. Fishman, W. H. (1987) *Adv. Cancer Res.* **48**, 1–35.
9. Fishman, L., Miyayama, H., Driscoll, S. G. & Fishman, W. H. (1976) *Cancer Res.* **36**, 2268–2273.
10. Donald, L. J. & Robson, E. B. (1974) *Ann. Hum. Genet.* **37**, 303–313.
11. Slaughter, C. A., Coseo, M. C., Cancro, M. P. & Harris, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1124–1128.
12. Millán, J. L., Beckman, G., Jeppsson, A. & Stigbrand, T. (1982) *Hum. Genet.* **60**, 145–149.
13. Fishman, W. H., Inglis, N. R., Stolbach, L. L. & Krant, M. J. (1968) *Cancer Res.* **28**, 150–154.
14. Nakayama, T., Yoshida, M. & Kitamura, M. (1970) *Clin. Chim. Acta* **30**, 546–548.
15. Chang, C. H., Angellis, D. & Fishman, W. H. (1980) *Cancer Res.* **40**, 1506–1510
16. Goldstein, D. J., Rogers, C. E. & Harris, H. (1982) *Clin. Chim. Acta* **125**, 63–75.
17. Wahren, B., Holmgren, P. Å. & Stigbrand, T. (1979) *Int. J. Cancer* **24**, 749–753.
18. Lange, P. H., Millán, J. L., Stigbrand, T., Vessella, R. L., Ruoslahti, E. & Fishman, W. H. (1982) *Cancer Res.* **42**, 3244–3247.
19. Wei, S. C. & Doellgast, G. J. (1981) *Eur. J. Biochem.* **118**, 39–45.
20. Millán, J. L. & Stigbrand, T. (1983) *Eur. J. Biochem.* **136**, 1–7.
21. Millán, J. L., Eriksson, A. & Stigbrand, T. (1982) *Hum. Genet.* **62**, 293–295.
22. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
23. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
25. Biggen, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci USA* **80**, 3963–3965.
26. Avrameas, S. (1969) *Immunochemistry* **6**, 43–52.
27. Millán, J. L., Stigbrand, T. & Jörnvall, H. (1985) *Int. J. Biochem.* **10**, 1033–1039.
28. Millán, J. L. (1987) *Nucleic Acids Res.* **24**, 10599.
29. Jeppsson, A., Wahren, B., Brehmer-Andersson, E., Silverswärd, C., Stigbrand, T. & Millán, J. L. (1984) *Int. J. Cancer* **34**, 757–761.
30. Tsavaler, L., Penhallow, R. C., Kam, W. & Sussman, H. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4529–4532.
31. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383.
32. Dynan, W. S. & Tjian, R. (1983) *Cell* **35**, 79–87.
33. Mellon, P., Parker, V., Gluzman, Y. & Maniatis, T. (1981) *Cell* **27**, 279–288.
34. Nordheim, A. & Rich, A. (1983) *Nature (London)* **303**, 674–678.
35. Jeppsson, A., Wahren, B., Stigbrand, T., Edsmyr, F. & Andersson, L. (1983) *Br. J. Urol.* **55**, 73–78.
36. McDicken, I. W., McLaughlin, P. J., Tromans, P. M., Luesley, D. M. & Johnson, P. M. (1985) *Br. J. Cancer* **52**, 59–64.
37. Nouwen, E. J., Hendrix, P. G., Dauwe, S., Eerdekens, M. W. & DeBroe, M. E. (1987) *Am. J. Pathol.* **126**, 230–242.
38. Jeppsson, A., Wahren, B., Millán, J. L. & Stigbrand, T. (1984) *Br. J. Cancer* **49**, 123–128.
39. Epenetos, A., Snook, D., Durbin, H., Johnson, P. M. & Taylor-Papadimitriou, J. (1986) *Cancer Res.* **46**, 3183–3191.