Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 224-231

# TESTINS ARE STRUCTURALLY RELATED TO THE MOUSE CYSTEINE PROTEINASE PRECURSOR BUT DEVOID OF ANY PROTEASE/ANTI-PROTEASE ACTIVITY

C. Yan Cheng[1], Ian Morris[2], and C. Wayne Bardin[1]

[1]The Population Council, Center for Biomedical Research, 1230 York Avenue, New York, New York 10021

[2]Department of Physiological Sciences, School of Biological Sciences, University of Manchester, Manchester M13 9PT, England

Received January 9, 1993

**SUMMARY:** Testin I (Mr 35,000) and testin II (Mr 37,000) (testins) are two structurally and immunologically related testicular proteins that are actively synthesized and secreted by Sertoli cells. Treatment of adult rats with busulfan to destroy germ cells selectively in the testis could lead to a significant increase in the testicular testins level; as the germ cells reappeared in the testis, the testicular testins level declined and returned to the normal level. These observations indicated that the testicular content of testins in the rat is inversely correlated to the number of germ cells. When the partial N-terminal amino acid sequences for testin I and testin II were compared with the existing protein data base at Protein Identification Resource, it was noted that they displayed remarkable identitiy with CTLA-2$\alpha$ and CTLA-2$\beta$, two novel molecules expressed in mouse activated T lymphocytes and mast cells, and the mouse cysteine proteinase proregion. When purified testins were assayed for the proteolytic and anti-protease activity using [$^{14}$C]-casein, it was noted that it possessed neither proteolytic nor anti-protease activity,suggesting that it is not functioning as a protease and/or protease inhibitor in the testis. 

Studies from this laboratory have shown that Sertoli cells cultured *in vitro* synthesize and secrete two structurally and immunologically related proteins designated testin I and testin II (1, 2). Both testin I and testin II are single-chained polypeptides (2). Partial N-terminal amino acid sequence analysis of testin I and testin II revealed that their N-terminal sequences are virtually identical except that testin II has three extra N-terminal amino acids of AAP compared to testin I (2). We have now extended these earlier studies and shown that the partial N-terminal amino acid sequences of testins share extensive identity with the mouse cysteine proteinase precursor proregion (3); as well as CTLA-2$\alpha$ and CTLA-2$\beta$, two novel molecules expressed in mouse activated T lymphocytes and mast cells (4). Since it has been shown that Sertoli cells, the major secretory component in the seminiferous epithelium, synthesize and secrete several proteases and protease inhibitors such as cathepsin L (5, 6) and $\alpha_2$-macroglobulin (7, 8) that are involved in the tissue remodelling in the seminiferous epithelium; we sought to determine if testins possess the biological activity of the cysteine proteinase.

Other recent studies from this laboratory have shown that the polarized secretion of testins by Sertoli cells in a bicameral culture system can be modulated by germ cells. It is noted that the apical and basal

0006-291X/93 $4.00

secretion of testins by Sertoli cells is inhibited and stimulated by germ cell-conditioned medium, respectively (9). We thus thought it pertinent to determine if the depletion of germ cells in the testis *in vivo* by a known reversible anti-spermatogenic alkane sulfonate, busulfan (butylene dimethanesulfonate) that alkylates DNA and selectively destroys spermatogonia (10, 11, 12), would affect the testicular testins level.

## MATERIALS AND METHODS

***Purification of testins from Sertoli cell-enriched culture medium.*** Testin I and testin II were purified from primary Sertoli cell-enriched culture medium essentially as previously described using sequential HPLC with anion-exchange (Mono Q, HR 10/16, 10 x 16 mm, i.d.) and chromatofocusing (Mono P, HR 5/20, 5 x 200 mm, i.d.) columns (1,2) except that the last gel permeation HPLC step was deleted and replaced with high performance electrophoresis chromatography (HPEC). The HPEC was used because of its superior recovery (greater than 95%) and fine resolution. HPEC was performed using an Applied Biosystems 230A HPEC system. Partially purified testin I or testin II obtained from the Mono P column was lyophilized, resuspended in 0.5 ml of double distilled water and concentrated to about 50 µl in an Amicon Microcon-10 microconcentrator. It was noted that this step of equilibration was crucial for the HPEC by reducing the salt concentration in the sample. For optimal separation and resolution of proteins, the total protein content should not exceed 100 µg total protein per run using a 3.5 x 50 mm (i.d.) SDS-polyacrylamide gel (10% *T*, total acrylamide concentration). The sample was then centrifuged at 15,000 g for 10 min to remove any precipitates and it was denatured in 50 µl of SDS-sample buffer [10 mM Tris-$PO_4$, pH 7.5 at 22C containing 0.5% SDS (w/v), 15% glycerol (v/v), and 0.002% bromophenol blue (w/v)] without 2-mercaptoethanol at 100C for 5 min. It was then loaded onto the gel and electrophoresed at constant current at 1.8 mA at 10C. Fractions of 220 µl each were collected using an elution buffer (75 mM Tris-$PO_4$, pH 7.4 at 22C) operated at a flow rate of 22 µl/min. The upper and lower running buffers were 75 mM Tris-$PO_4$, pH 7.4 at 22C containing 0.1% SDS (w/v); and 75 mM Tris-$PO_4$, pH 7.4 at 22C; respectively. Both buffers were operated at a flow rate of 1.5 ml/min and the eluents were monitored by UV absorbance at 280 nm using a variable UV wavelength detector built into the HPEC system. The chromatogram was recorded by a Hewlett Packard Paintjet printer interfaced to the HPEC system. It routinely took 12-16 hr to complete a run.

***$NH_2$-Terminal amino acid sequence analysis.*** Protein sequence analysis was performed using an Applied Biosystems 473A pulsed-liquid phase protein sequencer as previously described (7, 13). PHT(phenylthiohydantoin)-amino acids obtained from the Edman degradation cycles were automatically quantified by HPLC using a Brownlee PTH-C18 (2.1 x 220 mm, i.d.; particle size, 5 µm) HPLC column and an Applied Biosystems RP-18 guard cartridge (3.3 x 15 mm, i.d.). All data were acquired using a Mac IIcx workstation which was interfaced to the protein sequencer via an Applied Biosystems A/D Converter. Data analysis was performed using an Applied Biosystems Model 610A Data Analysis Program. The repetitive yield was about 96%.

***Protease and protease inhibitor assay.*** Protease and protease inhibitor were assayed using methyl-α-[$^{14}$C]-casein by established procedures as previously described (7). Protease was assayed by incubating increasing doses of protein (0.1 - 5 µg) in the absence or presence of protease inhibitor (10-20 µg protein) in 200 µl of protease buffer (0.025 M Tris, pH 7.4 at 22C, containing 3% NaCl; 1 mM EDTA and 5 mM cysteine were included in the protease buffer for papain). For protease inhibitor, it was assayed by incubating proteases (0.1-5 µg) in the presence or absence of protease inhibitors (10-20 µg protein) in 200 µl of protease buffer. For both assays, the tubes were incubated with [$^{14}$C]casein (10 µl, 0.5 µCi/ml) for 30 min at 22C. Thereafter, 200 µl of ice-cold trichloroacetic acid (20% w/v) and 300 µg of BSA (10 µl of 30 mg/ml stock) were added to each tube to precipitate the unreacted [$^{14}$C]casein. Following sedimentation at 12,000*g* for 2 min, hydrolyzed fragments in the supernatant were quantified by spectrometry using 5 ml of Beckman Ready Safe™ in a β-counter (LKB 1209 Rackbeta Liquid Scintillation Counter) at 28% counting efficiency.

***Treatment of rats with busulfan.*** Adult male Wistar rats (250-300 g body weight) were obtained from Charles River, housed in groups of 3-4 with free access to water and food *ad libitum*. Rats in groups of seven were injected intraperitoneally on day 0 with busulfan (10 mg/kg bw, Burroughs Wellcome Co.,

NC) or with the injection vehicle (control, arachis oils, 2 ml/kg bw) and decapitated at day 7, 21, 28, 42, 63, 84, and 105 for blood and tissue collection. Blood was collected from the jugular vein under light ether anesthesia, allowed to clot at 4C overnight and serum was obtained by centrifugation (3000 *g*, 20 min). Testicles were removed immediately, weighed, and stored at -20C. They were subsequently thawed and homogenized in 1:3 (w/v) in TG buffer [10 mM Tris, pH 7.4 at 22C, containing 10% glycerol (v/v)] and centrifuged at 45,000 *g* for 45 min at 4C to obtain the cytosol.

***Radioimmunoassay of testins.*** Since both testin I and testin II share common epitopes, antisera prepared against testin I quantify testin II as well. Radioimmunoassay to quantify the concentration of testins in the biological samples was performed as previously described (1, 2). The standard curve was calibrated using a pool of Sertoli cell-enriched culture medium designated EP-130 which was run in every assay in three replicates. All unknowns were also run in three replicates. It was noted that 1 µl of EP-130 contained 1.1 ng of testins (2). The minimal detectable dose and the 50% displacement were 0.05 µl eq/assay tube and 1.2 µl eq, respectively. The intra- and inter-assay coefficients of variation were 8 and 10%, respectively. All samples from a given experiment were radioimmunoassayed simultaneously to eliminate inter-assay variation. All data were computer analyzed as previously described (1,2).

***General methods.*** Protein concentration was determined using a Coomassie blue dye-binding assay (14). Analytical polyacrylamide gel electrophoresis in the presence of SDS was performed according to the methods of Laemmli (15).

## RESULTS

***Structural and biological characterization of testins.*** Purified testin I (Fig. 1A) and testin II (Fig. 1B) were eluted from a 10% *T* SDS-polyacrylamide gel in the HPEC system and their purity was

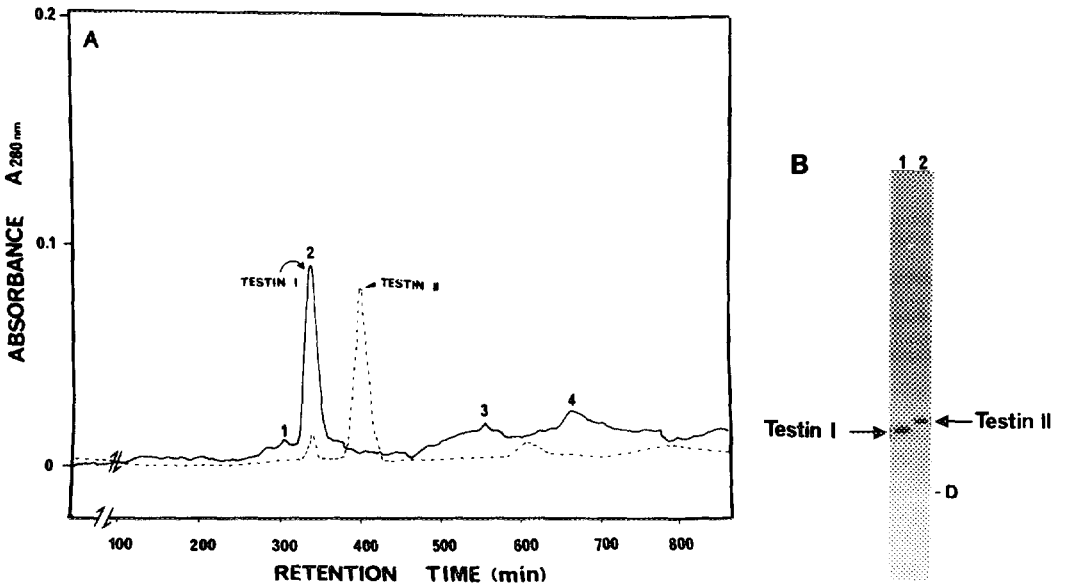


**Fig. 1.** Purification of testin I and testin II by HPEC. **(A)** Partially purified testin I and testin II from the chromatofocusing HPLC step were pooled and prepared for HPEC separately as described under **Materials and Methods.** Electrophoresis was performed for 14 hr at 10C using a 3.5 x 50 mm (i.d.) 10% *T* SDS-polyacrylamide rod gel. Testin I (solid line) and testin II (broken line) were eluted with different retention times as shown. **(B)** An aliquot of 5 µl (about 0.3 µg protein) from the pooled fractions under the peak containing either purified testin I or testin II was resolved by SDS-PAGE onto a 10% *T* SDS-polaycrylamide gel and the gel was silver stained.

confirmed by silver stained SDS-polyacrylamide gel as shown in Fig. 1C. It was noted that their partial N-terminal amino acid sequences were identical except that testin II has three extra N-terminal amino acids of AAP. Comparison of these sequences with the existing protein data base at Protein Identification Resource (R32.0) and GenBank (R72.0) indicated that they displayed remarkable similarity with CTLA-2α, CTLA-2β, and the proregion of mouse cysteine protease (Fig. 2). When two different batches of purified testin I and testin II were bioassayed for their proteolytic activity, it was shown that neither of these proteins possess the proteolytic activity to cleave [$^{14}$C]-casein (Table 1). It was also noted that neither testin I nor testin II possess any anti-protease activity by inhibiting the proteolytic effect of trypsin on [$^{14}$C]-casein (Table 1). To eliminate the possibility that the protein was denatured during the HPEC step, we had used another batch of purified protein isolated by sequential HPLC on anion-exchange, chromatofocusing, and gel permeation HPLC as previously described (1, 2) for the protease and anti-protease assay; it was noted that this other batch of purified protein also did not display any proteolytic activity indicating that testins are neither proteases nor protease inhibitors.

***Relationship of testicular testins level and germ cells.*** When adult rats were treated with a single dose of busulfan, a significant decrease in the testicular weight was noted (Fig. 3A). However, it had no apparent effect on the body weight of the animals (data not shown). The maximal decline in testicular weight was noted between day 42 and 63; thereafter, the testicular weight returned to normal level by day 105 (Fig. 3A). The depletion of germ cells in the seminiferous epithelium by the busulfan treatment was monitored histologically as previously described (12); it was noted that by day 42, there was no significant change in the testicular sperm count consistent with the early loss of immature germ cells. By day 63, there was a significant decline in the number of more mature germ cells. By day 105, the testicular sperm count was about 60% of the control level. When the concentration and the testicular content of testins were quantified, it was noted that there was a significant increase in the testins concentration (Fig. 3B) and its testicular content (Fig. 3C) between days 21 and 28, indicating an early

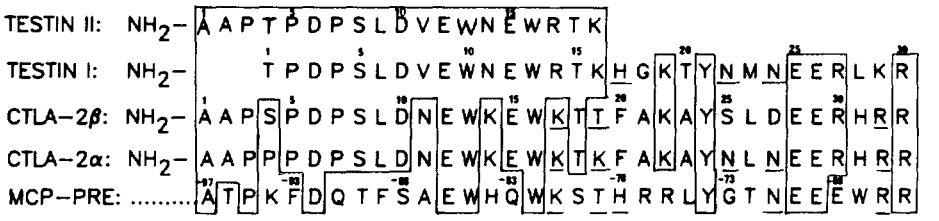


**Fig. 2.** Comparison of the N-terminal amino acid sequences between testin I, testin II, CTLA-2α, CTLA-2β, and mouse cysteine proteinase. The N-terminal amino acid sequences for CTLA-2α, CTLA-2β, the proregion of mouse cysteine proteinase (MCP-PRE) were deduced from the nucleotide sequences (3, 4). The sequences of testins were determined by direct amino acid sequence analysis using an Applied Biosystems 473A pulsed-liquid phase protein sequencer as described in **Materials and Methods**.

**TABLE 1: STUDY ON THE EFFECTS OF HYDROLYSIS OF [$^{14}$C]CASEIN BY PROTEOLYGIC ENZYMES AND TESTINS WITH OR WITHOUT PROTEASE INHIBITORS[a]**

| Enzyme (protein) | | Protease inhibitor | Hydrolysis products (cpm) |
|---|---|---|---|
| Trypsin: | 0.1 μg | - | 8900±516 |
| | 0.1 μg | soybean trypsin inhibitor (10 μg) | 1497±123 |
| | 1.0 μg | - | 11439±387 |
| | 1.0 μg | soybean trypsin inhibitor (10 μg) | 1449±39 |
| | 1.0 μg | testin I (10 μg) | 10343±330 |
| | 1.0 μg | testin II (10 μg) | 10120±520 |
| Papain | 1.0 μg | - | 9345±279 |
| | 1.0 μg | $\alpha_2$-macroglobulin (20 μg) | 1489±112 |
| | 5.0 μg | - | 13294±1232 |
| | 5.0 μg | $\alpha_2$-macroglobulin (20 μg) | 2179±80 |
| Testin I | 0.1 μg | - | 1332±23 |
| | 0.5 μg | - | 1382±90 |
| | 1.0 μg | - | 1430±16 |
| | 5.0 μg | - | 1470±109 |
| Testin II | 0.1 μg | - | 1271±95 |
| | 5.0 μg | - | 1674±150 |

[a]The proteolytic assays were performed as described under **Materials and Methods** using purified proteins. Each number represents the result of triplicate determinations of a given experiment. These experiments were repeated twice using two separate batches of purified testin I and testin II, and similar results were obtained in each experiment.

loss of immature germ cells would elicit a significant synthesis and/or secretion of testins by Sertoli cells. However, when the germ cells reappeared in the testis, the testicular concentration and the testicular content of testins returned to the control level (Fig. 3 B, C).

## DISCUSSION

Using a monospecific polyclonal antibody prepared against testin I and immunofluorescence microscopy, it was noted that testins were localized between Sertoli-Sertoli junctional complexes below and above the preleptotene spermatocytes in Stage VIII-IX seminiferous tubules; whereas in Stage VII seminiferous tubule, testins was observed over Type $A_1$ spermatogonia and late spermatids (16). In the epididymis, testins were localized at the apices of the epithelial cells adjacent to the epididymal lumen at the sites of known junctional complexes suggesting that these proteins might be a component of the tight junctional complexes in the reproductive tract (16). Many of the known junctional proteins possess an apparent Mr much higher than testins; these include cingulin-140 (Mr 140,000), cingulin-108 (Mr 108,000) (17), and ZO-1 (Mr, 225,000) (18). However, none of these molecules share amino acid sequence homology with testins suggesting that testins are indeed novel components of the junctions between cells in the testis,

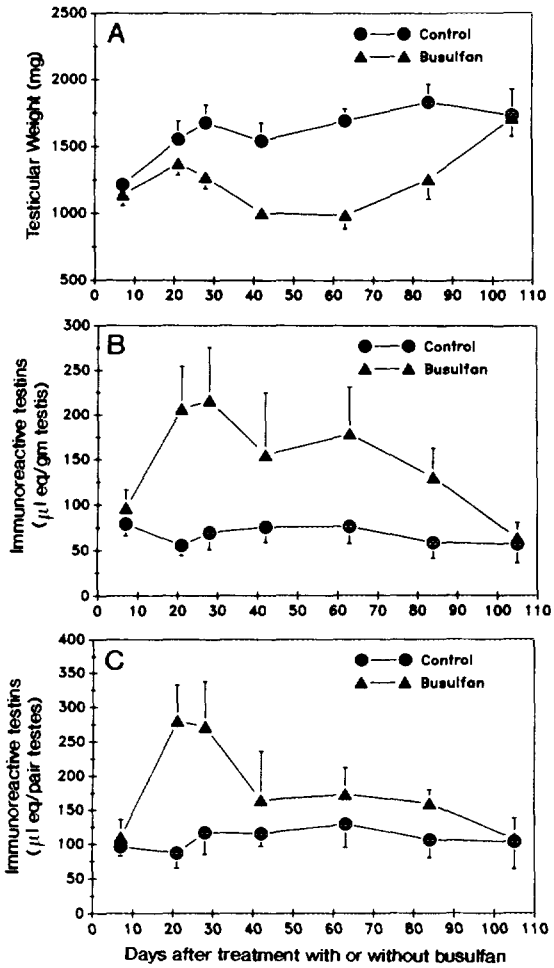


**Fig. 3.** Changes of the testicular weight **(A)**, the concentration **(B)**, and the content **(C)** of testins in the testis following a single dose of busulfan treatment in adult rats. A group of 7 adult Wistar rats was used for each data point and the animals were treated as described in **Materials and Methods.** The levels of testins in the samples were determined by RIA in adult rats at 0 (control), 7, 21, 28, 42, 63, 84, and 105 days. All samples were quantified in a given radioimmunoassay in three replicates.

epididymis, and possibly other organs. In the present study, we have shown that testins share sequence identity with CTLA-2α, CTLA-2β, and the proregion of the mouse cysteine protease; however, it does not have the proteolytic activity of this latter molecule. It is noted that the functional significance of the CTLA-2α and CTLA-2β in the activated T lymphocytes and in mast cells are not known since it remains to be determined if these cells indeed actively synthesize and secrete these molecules either *in vitro* or *in vivo*.

Sertoli cells are the major phagocytic component in the seminiferous epithelium that are responsible for the disposal of residual bodies and degenerating germ cells (19-21). This phagocytic function is not

under hormonal control but by some of the same elements that are potent activators of macrophages (22) including dead cells, adjuvants, latex beads, barium sulfate, and microbial products. Thus, Sertoli cells and macrophages are similar in some functional aspects. Since there has an extensive tissue remodeling in the seminiferous epithelium behind the blood-testis barrier, it is logical to envision that Sertoli cells would secrete proteins that are involved in these events. In this study, we have shown that testins share amino acid sequence identity with two novel molecules designated CTLA-2$\alpha$ and CTLA-2$\beta$ that are expressed in activated T lymphocytes and in mast cells demonstrated by Northern blots (4). It has been shown that T lymphocytes secrete a number of novel proteinases such as serine esterases upon activation (23-25). Both testins, CTLA-2$\alpha$ and CTLA-2$\beta$ share sequence identity with the pro-region of the mouse serine protease (3, 4). Other studies have shown that a number of proteases are synthesized as precursors that consist the enzymatic moiety and an amino-terminal extension called pro-region (26). Prior to their secretions, the proregion will be cleaved enzymatically leading to the acquisition of proteolytic activity in the enzymatic moiety and that the proregions possess an inhibitory action to the corresponding proteolytic activity in the enzymatic moiety (27, 28). However, testins did not have any proteolytic nor anti-protease activity suggesting that they are not related to the serine protease functionally. In view of their structural relationship to molecules expressed in activated T lymphocytes, we speculate that testins may play a role in Sertoli cell activation in the seminiferous epithelium as well as a component of the junctional complexes in the testis.

## ACKNOWLEDGMENTS

This work was supported by a grant from National Institutes of Health (HD-13541). Ovine FSH was kindly provided to this laboratory by the National Hormone and Pituitary Program, NIDDK, NIH and the National Hormone and Pituitary Program, University of Maryland School of Medicine for the preparation of Sertoli cell-enriched culture medium for the purification of testins (Award numbers: 31887 and 32751).

## REFERENCES

1. Cheng CY and Bardin CW (1987) J. Biol. Chem. 262:12768-12779.
2. Cheng CY, Grima J, Stahler MS, Lockshin RA and Bardin CW (1989) J. Biol. Chem. 264:21386-21393.
3. Portnoy DA, Erickson AH, Kochan J, Ravetch JV and Unkeless JC (1986) J. Biol. Chem. 261:14697-14703.
4. Denizot F, Brunet JF, Roustan P, Harper K, Suzan M, Luciani MF, Mattei MG and Golstein P (1989) Eur. J. Immunol. 19:631-635.
5. Cheng CY, Zwain I, Grima J and Bardin CW (1991) Structure and functions of Sertoli cell proteins. In: *Comparative Spermatology, 20 Years After. Proceedings of the VI International Congress on Spermatology.* B. Baccetti Ed. New York, Raven Press, pp. 617-624.
6. Erickson-Lawrence M, Zabludoff SD and Wright WW (1990) J. Cell. Biol. 111:107a.
7. Cheng CY, Grima J, Stahler MS, Guglielmotti A, Silvestrini B and Bardin CW (1990) Biochemistry 29:1063-1068.

8. Stahler MS, Schlegel P, Bardin CW, Silvestrini B and Cheng CY (1991) Endocrinology 128:2805-2814.
9. Grima J, Pineau C, Bardin CW and Cheng CY (1992) Mol. Cell. Endocrinol. 89:127-140.
10. Jackson H, Partington M and Fox BW (1962) Nature 194:1184-1185.
11. Lee IP (1983) Am. J. Ind. Med. 4:135-147.
12. Morris ID, Bardin CW, Musto NA, Thau RB and Gunsalus GL (1987) Int. J. Androl. 10:691-700.
13. Cheng CY (1990) Biochem. Biophys. Res. Commun. 167:1393-1399.
14. Macart M and Gerbaut L (1982) Clin. Chim. Acta 122:93-101.
15. Laemmli UK (1970) Nature 227:680-685.
16. Zong SD, Bardin CW, Phillips D and Cheng CY (1992) Biol. Reprod. 47:568-572.
17. Citi S, Sabanay H, Jakes R, Geiger B and Kendrick-Jones J (1988) Nature 333:272-276.
18. Stevenson BR, Siliciano JD, Mooseker MS and Goodenough DA (1986) J. Cell Biol. 103:755-766.
19. Chemes H (1986) Endocrinology 119:1673-1681.
20. Niemi M and Kormano M (1965) Anat. Rec. 151:159-170.
21. Carr I, Clegg EJ and Meek GA (1968) J. Anat. 102:501-509.
22. Oppenheim JJ, Mizel SB and Meltzer MS (1979) in: Biology of Lymphokines, Eds. Cohen S, Pick E and Oppenheim JJ, New York, Academic Press, pp. 291-302.
23. Brunet JF, Dosseto M, Denizot F, Mattei MG, Clark WR, Haqqi TM, Ferrier P, Nabholz M, Schmitt-Verhulst AM, Luciani MF and Golstein P (1986) Nature 322:268-271.
24. Lobe CG, Finlay BB, Paranchych W, Paetkau VH and Bleackley RC (1986) Science 232:858-861.
25. Bond JS and Butler PE (1987) Ann. Rev. Biochem. 56:333-345.
26. Neurath H (1984) Science 224:350-357.
27. San Segundo B, Martinez MC, Vilanova M, Cuchillo CM and Aviles FX (1982) Biochim. Biophys. Acta 707:74-82.
28. Evin G, Devin J, Castro B, Menard J and Corvol P (1984) Proc. Natl. Acad. Sci. USA 81:48-52.