Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 351-358

A MICROSEQUENCING APPROACH TO IDENTIFY PROTEINS WHICH APPEAR TO INTERACT WITH THYROTROPIN IN RAT FRTL-5 THYROID CELLS

Takashi Akamizu, Motoyasu Saji, and Leonard D. Kohn

Section on Cell Regulation, Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Received June 8, 1990

In order to resolve questions concerning the in situ structure of the thyrotropin (TSH) receptor, [^{35}S]methionine-labeled thyroid cell preparations were detergent solubilized and proteins exhibiting TSH-dependent binding to TSH-Sepharose were identified. Two such proteins, 43 and 70 kd, are identified in this report as gamma-actin and a member of the heat shock 70 protein family, respectively, based on the microsequence of two peptides from each. Identification of the former was confirmed by Western blotting and immunostaining using anti-actin, the latter by its ability to bind [^{32}P]ATP, a characteristic feature of this family of proteins. The results suggest that TSH-cross linking reports defining TSH receptor subunits should be viewed with caution in the absence of comparative sequence data; consideration must, however, be given to the existence of receptor associated proteins.

Despite recent studies which have cloned the thyrotropin (TSH) receptor, protein M_r approximately 87,000 (1-5), its in-situ structure remains unclear because of the multiplicity of proteins which appear to interact with TSH. Studies using nondenaturing conditions have identified TSH-binding thyroid proteins or protein complexes with estimated molecular weights of approximately 500, 300, and 150 kd (6, 7). Investigators using TSH cross-linking or photoaffinity-labeling and gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) (8, 9) have identified 50-70, 30-45, and 15-25 kd components. The latter studies resulted in a postulated TSH receptor structure composed of 2 or 3 subunits. The problem of multiple TSH-binding proteins coupled to different signals or actions was not eliminated nor was the possibility there are TSH

Abbreviations: TSH, thyroid stimulating hormone; Hsp70, heat shock protein 70 kd; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; BSA, crystalline bovine serum albumin.

0006-291X/90 $1.50

receptor associated proteins identified by the procedures in use. The present report describes the application of a microsequencing approach to resolve these questions.

MATERIALS AND METHODS

Cell Culture - The FRTL-5 rat thyroid cells (ATCC no. CRL 8305, Interthyr Research Foundation, Inc.) are a continuous line of functioning cells derived from normal Fisher rats (10). The isolation, growth, and basic characteristics of these cells have been previously described (10, 11).

Biosynthetic Labeling and Detergent Solubilization of FRTL-5 cells -Dishes containing confluent cells grown in complete medium containing TSH (6H) (10, 11) were washed twice with methionine-free Dulbecco's modified essential medium containing 5% serum and a 5H medium mixture (no TSH); incubated 14 hours at 37°C in the same medium containing 100 μCi/ml [^{35}S]methionine; and washed twice with Hanks' balanced salt solution. Crude plasma membrane (6) preparations or the cells themselves were solubilized, 30 min at 0°C, by the direct addition of 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Nonidet P-40 (NP-40). In both cases, the solubilized protein mixture was centrifuged at 100,000xg for 60 min at 0-4°C before the supernatant solution was adsorbed to TSH-Sepharose.

Affinity Chromatography on TSH-Sepharose and SDS-PAGE - TSH-Sepharose was prepared as previously described (12); control columns of Tris-Sepharose were made by the same procedure but 0.1M Tris-HCl, pH 8.0, replaced the TSH. Solubilized, radiolabeled thyroid cell extracts were incubated with TSH- or Tris-Sepharose at 0-4°C.

To identify proteins exhibiting TSH-dependent binding to TSH Sepharose, equal volumes of TSH-Sepharose were incubated with extracts in the presence or absence of $1x10^{-6}$M TSH (a 10-fold excess by comparison to the amount coupled to the Sepharose, which was approximately $1x10^{-7}$M TSH) or 1% bovine serum albumin (BSA). Incubations were for 60 min before nonbound proteins were removed by microfuge centrifugation; the TSH-Sepharose was then washed five times with solubilizing buffer containing only 0.1% NP-40. Bound proteins were sequentially eluted with aliquots of a high salt (wash buffer plus 2M NaCl) and low pH (100 mM glycine-HCl, pH 2.5, containing 0.1% NP-40 and 2mM PMSF) buffer.

In experiments where TSH was the eluting agent, detergent extracts were adsorbed to TSH-Sepharose using the same batch procedure as above, washed, and eluted with washing buffer containing $5x10^{-6}$M TSH or 1% BSA.

Preparative experiments used TSH-Sepharose columns (10 ml), a peristaltic pump (10 ml/h), and a recirculating procedure which cycled the extract at least 10 times. Columns were washed (washing buffer) until fewer than 50 cpm/ml were eluted. Adsorbed proteins were removed by sequential exposure to the wash, high salt, and low pH buffers as noted above.

SDS-PAGE used a discontinuous buffer system (13). Autoradiography used an enhancer solution (Enlightening) as necessary. Alternatively, proteins on the gels were transblotted (Bio-Rad apparatus) onto PVDF membranes (14) and microsequenced or immunostained.

Immunostaining and Microsequence Analysis - Immunostaining used procedures described by, and reagents obtained from, Bethesda Research Laboratories. Microsequencing was performed after eluates from TSH-Sepharose columns were subjected to preparative SDS-PAGE

and overnight autoradiography to identify individual proteins. After a protein was excised from the gel, the gel slice was placed in the sample well of a second preparative SDS-PAGE gel and digested with Staphylococcus V8 protease (15). Individual peptides obtained after SDS PAGE were electroblotted, stained, and placed directly in an Applied Biosystems 470 gas-phase sequencer (14, 16). Protein sequences were evaluated using a VAX750 computer, the National Biomedical Research Foundation protein data bank, and the program Wordsearch (17).

Other Procedures - Membrane fractionation with Triton X-114 (17) and assays of cAMP levels in FRTL-5 cells were measured as described (11). Statistical analysis was performed using the student t test. The photoaffinity-labeling procedure of Evans et al. (19) was used to measure binding of ATP to the 70 kd protein.

Materials - Bovine TSH was a purified preparation (6) or was purchased from Sigma. [^{35}S]Methionine (Tran^{35}S-label, 1105 Ci/mmol) and [^{32}P]azido ATP (8-azidoadenosine-5'-triphosphate[gamma-^{32}P], 14.3 Ci/mmol) were from ICN Radiochemicals. [^{14}C]Methylated standard proteins, and cAMP radioimmunoassay kits were from Dupont-New England Nuclear. PVDF membranes were from Millipore.

RESULTS AND DISCUSSION

Solubilized, radiolabeled extracts from whole thyroid cells or membrane preparations were applied to TSH-Sepharose in the

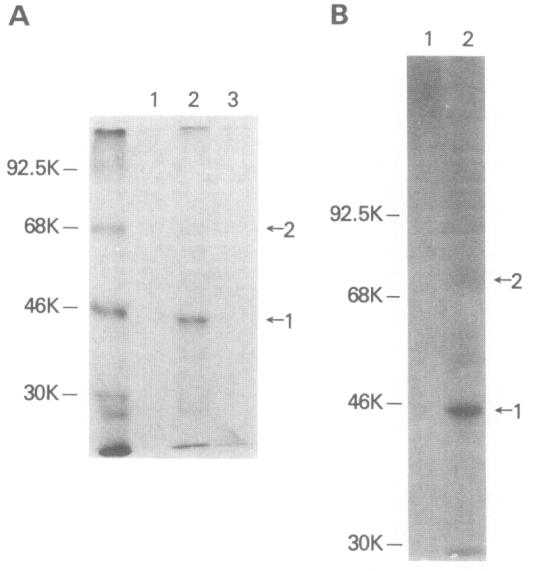
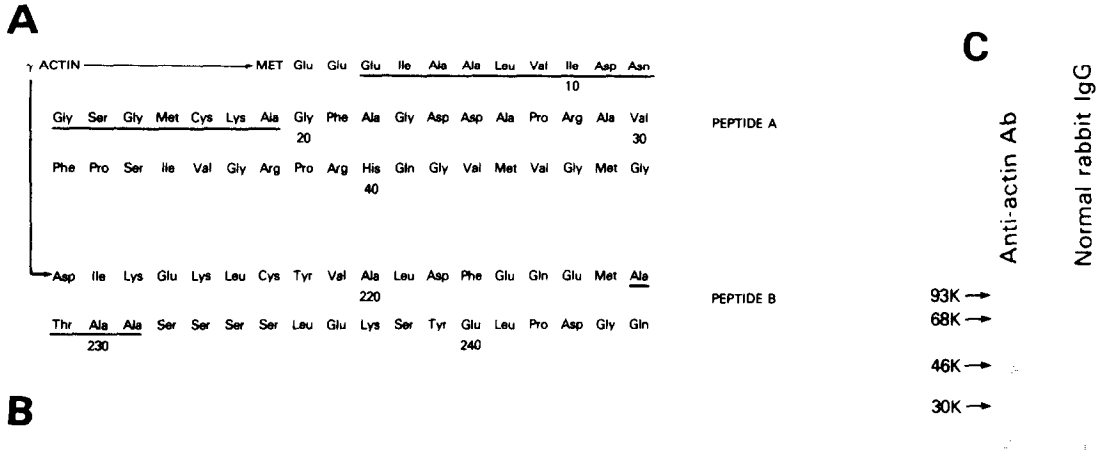


Figure 1. Identification of proteins exhibiting TSH-dependent binding to TSH-Sepharose. (A) SDS-PAGE analysis of [^{35}S]labeled thyroid FRTL-5 cell membrane proteins adsorbed to TSH-Sepharose in the presence of 1% albumin and eluted in the pH 2.5 eluate (lane 2) but not adsorbed to Tris-Sepharose (lane 1) or to TSH-Sepharose in the presence of $1x10^{-6}$M free TSH (lane 3). (B) SDS-PAGE analysis of solubilized [^{35}S]labeled FRTL-5 thyroid cell proteins adsorbed to Tris-Sepharose (lane 1) or TSH-Sepharose (lane 2) and eluted with $5x10^{-6}$M free TSH. Autoradiography was for 48 hours in both cases.

presence or absence of purified TSH and non-bound material removed by washing. Proteins shown to exhibit TSH-dependent binding were eluted by sequential treatments with high salt or low pH buffers. In a typical experiment, the two fractions contained 0.025% and 0.0025%, respectively, of the radioactivity incorporated into total cell protein whether whole cells or membrane fractions were used. The two most prominent proteins (1 and 2), M_r 43 and 70 kd, respectively, in the low pH eluate did not bind to Tris-Sepharose (Fig. 1A, lane 1) nor to TSH-Sepharose when a 10-fold excess of free TSH was present during the period of adsorption of the extract to the TSH-Sepharose (Fig. 1A, lane 3). In contrast, their binding to TSH-Sepharose was not prevented when 1% albumin was present during the adsorption period to the column (Fig. 1A, lane 2). When the TSH-Sepharose was eluted with TSH as described by Remy et al. (20), the 43 kd and 70 kd proteins were both present in the TSH eluate (Fig. 1B, lane 2, proteins 1 and 2); again, 1% albumin did not duplicate the effect of TSH (Fig. 1B, lane 1).

Larger quantities of the 43 and 70 kd proteins which appeared to exhibit TSH-dependent binding to TSH-Sepharose were obtained by



Residue No.	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
peptide A			Glu	Ile	Ala	Ala	Leu	X	Ile	Asp	Asn	Gly	X	Gly	Met	X	Lys	Ala
human γ actin	Glu	Glu	Glu	Ile	Ala	Ala	Leu	Val	Ile	Asp	Asn	Gly	Ser	Gly	Met	Cys	Lys	Ala
human β actin			Asp	Ile	Ala	Ala	Leu	Val	Val	Asp	Asn	Gly	Ser	Gly	Met	Cys	Lys	Ala
Rat β actin			Asp	Ile	Ala	Ala	Leu	Val	Val	Asp	Asn	Gly	Ser	Ala	Met	Cys	Lys	Ala

Figure 2. Identification of the 43 kd protein as gamma-actin. (A) Comparison of the amino acid sequence of peptides A and B (underlined) from the V8 protease digest of the 43 kd protein (Fig. 1) with the sequence of gamma-actin. (B) The sequence of peptide A compared with gamma and ß-actin sequences. (C) Immunostaining of the 43 kd protein with rabbit anti-actin or normal rabbit IgG after the pH 2.5 eluate from TSH-Sepharose (Fig. 1) was Western blotted and immunostained using a biotin-strepavidin-peroxidase detection system.

preparative SDS-PAGE and two peptides from each were microsequenced after protease digestion, since each was found to be N-terminally blocked. The two peptides from the 43 kd protein (A and B) were identical in sequence to residues 4-19 of gamma actin (Fig. 2A); gamma-actin can be distinguished from ß-actin by the presence of glutamic and isoleucine residues at position 4 and 10, respectively, of the actin sequence (Fig. 2B). Residue 15 of rat ß-actin is an alanine residue whereas the comparable residue in the peptide A is a glycine, as in human gamma-actin (Fig. 2B). The identification of the 43 kd protein as an actin protein was confirmed by its reaction with anti-actin after Western blotting and immunostaining (Fig. 2C). A portion of the 43 kd protein pool

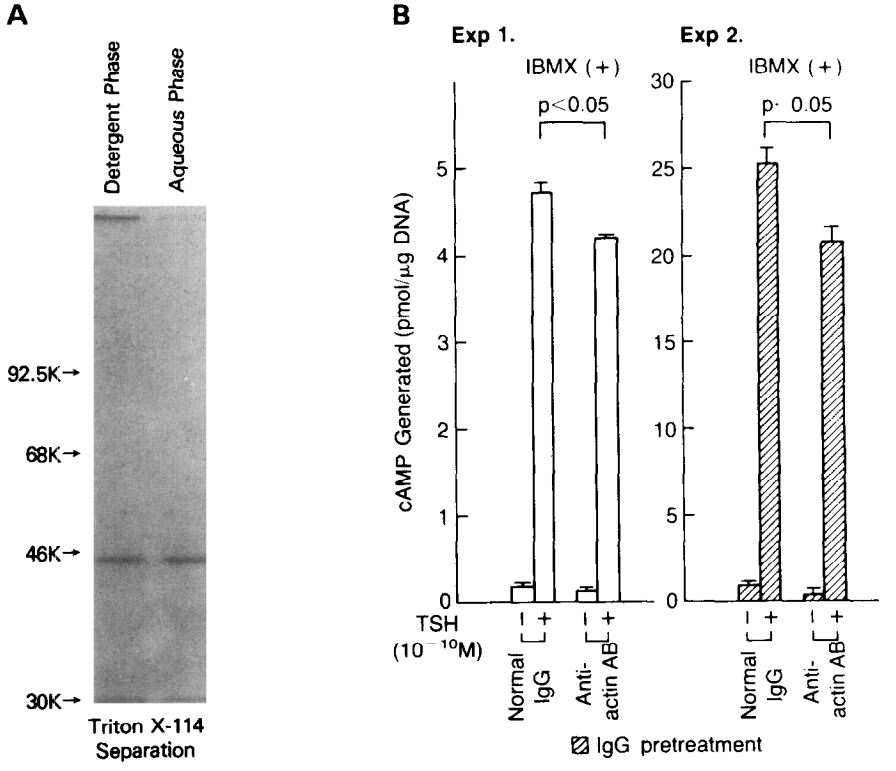


Figure 3. (A) Autoradiographic (Fig. 2C) identification of a portion of the 43 kd protein in the detergent phase after Triton X-114 temperature-induced phase separation. (B) Ability of rabbit anti-actin IgG to inhibit TSH-elevated cAMP levels in FRTL-5 thyroid cells compared to the action of normal rabbit IgG. Exp. 1 and Exp. 2 differ in two respects: a) different subclones of FRTL-5 thyroid cells (K and F1, respectively, in Exp. 1 and 2) and b) the anti-actin or normal IgG was added with (Exp. 1), or 90 min before (Exp. 2), the addition of $1x10^{-10}$M TSH. Values are the mean ± SE of triplicate assays, performed in duplicate, on three different batches of cells. The inhibition was significant ($p< 0.05$).

does appear to be an intrinsic protein of thyroid membranes. Thus, it is recovered in the detergent as well as aqueous phase (Fig. 3A) after thyroid membrane preparations are extracted with Triton-X 114, an agent which extracts intrinsic membrane proteins into the detergent phase (18). In addition, rabbit anti-actin, but not normal rabbit immunoglobulin preparations, can cause a small but significant ($p<0.05$) inhibition of the ability of TSH to increase cAMP levels in intact rat FRTL-5 thyroid cells (Fig. 3B).

The two peptides of the 70 kd protein exhibited a significant homology with sequences of several proteins in the heat shock 70 protein family (Fig. 4A). Fragment C, for example, exhibited 58-74% homology with the three individual members of the family presented in Figure 4 and a nearly 95% homology by comparison to

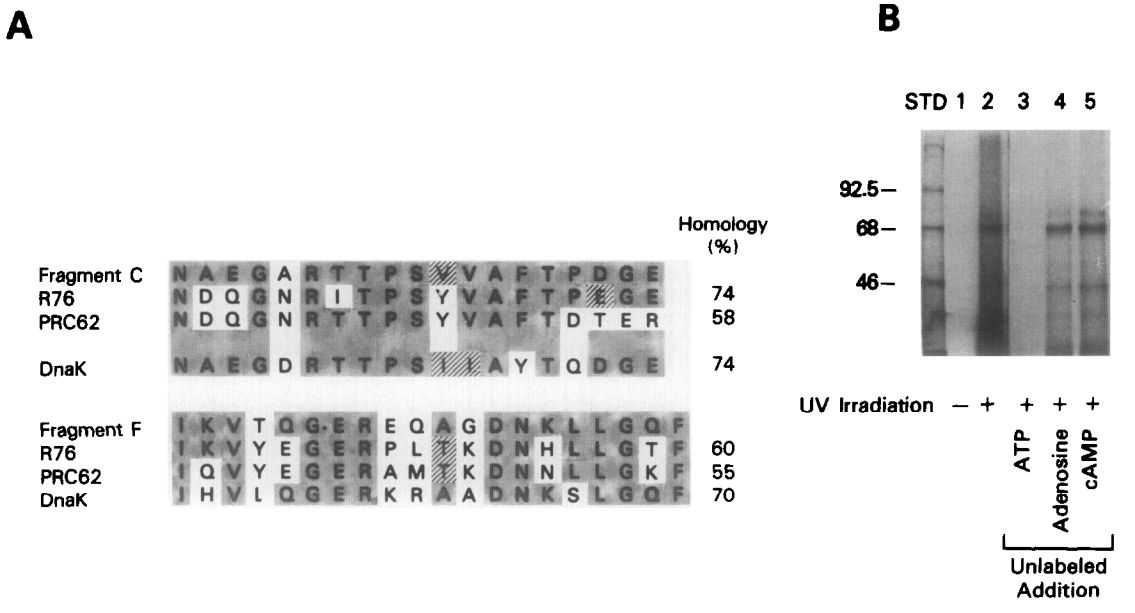


Figure 4. Identification of the 70 kd protein as a member of the Hsp70 protein family. (A) Comparison of the amino acid sequence of peptides C (Top) and F (bottom) from the V8 protease digest of the 70 kd protein (Fig. 1) with the sequence of three members of the Hsp70 protein family: R76 from rat liver, PRC62 from rat pheochromocytoma, and DnaK Hsp 70 from bacteria. Solid boxes denote residues which are identical; cross-hatched boxes denote residues considered similar, i.e. which can interchange without modifying the biological activity of a protein. Open boxes denote differences. (B) Ability of the 70 kd protein to bind ATP. The pH 2.5 eluate from the TSH-Sepharose (Fig. 1) was incubated with 8-N_3-[gamma^{32}P]ATP and not exposed (-) or exposed (+) to uv light in the absence or presence of unlabeled ATP (2 mM), adenosine (20 mM), and cAMP (20 mM) to evaluate the specificity of the binding with respect to ATP and the photolabeling procedure. The arrows denote the 70 kd protein; standards are [^{14}C]methylated proteins.

the entire group, i. e. only residue 5, alanine, is not identical or very similar to a residue in one of the members of the family. As expected for heat shock 70 proteins (21), the 70 kd protein does bind ATP when incubated with [^{32}P]azido ATP in the presence, but not absence, of ultraviolet light (Fig. 4B). The binding of the [^{32}P]azido ATP is prevented by unlabeled ATP but not by unlabeled adenosine or cAMP (Fig. 4B) nor by AMP or ADP (data not shown).

Given the recent cloning and sequencing of the TSH receptor (1-5), the present studies suggest that reports defining a subunit structure for the TSH receptor based on the ability of different sized proteins to interact with TSH (8, 9) should be viewed cautiously. In contrast, the possibility that these are TSH receptor associated proteins which account for the high molecular weight TSH-binding proteins in studies under nondenaturing conditions (6, 7) is not excluded and deserves further study. Thus, consistent with present results, gamma actin, the membrane-associated cytoskeleton form of actin (22), has been linked to the TSH and the TSH receptor by Avivi et al. (23) in studies of membrane clustering and endocytosis of TSH into FRTL-5 thyroid cells. Further, although Hsp70 can bind to many proteins by nonspecific hydrophobic interactions (21) which are yet to be excluded, not only has its specific association with the progesterone receptor now been demonstrated (24) but also (a) Hsp70 is important in the immune response (25); (b) Hsp70 is argued to be important in the ability of infectious agents such as Yersinia enterocolitica to induce autoimmune thyroid disease (26); and (c) TSH can inhibit the reaction of Yersinia enterocolitica antibodies with thyroid proteins, including Hsp70 (27). The Hsp70/TSH-TSH receptor relationship appears, therefore, to be important to the development or expression of autoimmune Graves' disease.

REFERENCES

1. Akamizu, T., Ikuyama, S., Saji, M., Kosugi, S., Kozak, C., McBride, W. O., and Kohn, L. D. (1990) Proc. Natl. Acad. Sci. USA, in press.
2. Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J. E. and Vassart, G. (1989) Science 246, 1620-1622.
3. Nagayama, Y., Kaufman, K.D., Set, P., and Rapoport, B. (1989) Biochem. Biophys. Res. Commun. 165, 1184-1190.
4. Libert, F., Lefort, A., Gerard, C., Parmentier, M., Perret, J., Ludgate, M., Dumont, J. E. and Vassart, G. (1989) Biochem. Biophys. Res. Commun. 165, 1250-1255.
5. Misrahi, M., Loosfelt, H., Atger, M., Sar, S., Guiochon-Mantel, A., and Milgrom, E. (1990) Biochem. Biophys. Res. Commun. 166, 394-403.

6. Tate, R. L., Holmes, J. M., Kohn, L. D., and Winand, R. J. (1975) J. Biol. Chem. 250, 6527-6533.
7. Drumond, R. W., McQuade, R., Grunwald, R., Thomas Jr., C. G., and Nayfeh, S. M. (1982) Proc. Natl. Acad. Sci. USA 79, 2202-2206.
8. Rees Smith, B., Rickards, C. R., Davies-Jones, E., Kajita, Y., Buckland, P. R., Creagh, F. M., Howells, R. D., Hashim, F., Parkes A. B., and Petersen, V. B. (1985) J. Endocrinol. Invest. 8, 175-182.
9. Genneck, S. E., Thomas Jr., C. G., and Nayfeh, S. M. (1987) Endocrinology 121,2119-2130.
10. Ambesi-Impiombato, F. S. (1986) U.S. Patent no 4,608,341.
11. Kohn, L. D., Valente, W. A., Grollman, E. F., Aloj, S. M., and Vitti, P. (1986) U.S. Patent no 4,609,622.
12. Tate, R. L., Winand, R. J., and Kohn, L. D. (1974) Methods Enzymol. 34, 692-695.
13. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685.
14. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035-10038.
15. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102-1106.
16. Johe, K. K., Smith, A. J., Vengelen-Tyler, V., and Blumenfeld, O. O. (1989) J. Biol. Chem. 264, 17486-17493.
17. Zarrilli, R., Oates, E.L., McBride, O.W., Lerman M.I., Chan, J.Y., Santisteban, P., Ursini, M.V., Notkins, A.L. and Kohn, L.D. (1989) Mol. Endocrinol. 3: 1498-1508.
18. Bordier, C. (1981) J. Biol. Chem. 256, 1604-1607.
19. Evans, R. K., Haley, B. E., and Roth, D. A. (1985) J. Biol. Chem. 260, 7800-7804.
20. Remy, J. J., Salamero, J., and Charreire, J. (1987) Endocrinology 121, 1733-1741.
21. Lindquist, S. (1986) Ann. Rev. Biochem. 55, 1151-1191.
22. Erba, H. P., Gunning, P., and Kedes, L. (1986) Nuc. Acids Res. 14, 5275-5294.
23. Avivi, A., Tromantono, D., Ambesi-Impiombato, F. S., and Schlessinger, J. (1982) Mol. Cell Endocrinol. 25. 55-71.
24. Kost, S. L., Smith, D. F., Sullivan, W. P., Welch, W. J., and Toft, D. O. (1989) Mol. Cell Biol. 9, 3829-3838.
25. Pardue, M. L. (1988) Genes & Development 2, 783-785.
26. DeGroot, L. J. and Quintans, J. (1989) Endocr. Rev. 10, 537-562.
27. Wenzel, B. E., Heufelder, A. E., Franke, T., Heesemann, H. B., Mehta, W., and Dillmann W. (1990) Proceedings American Endocrine Society 72nd Annual Meeting, Abstract 1, p. 25.