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Heterogeneous distribution of nuclear triiodothyronine receptors in liver and preadipose cells as evidenced by their reactivity to antibodies against different protein products of *erb* A oncogenes

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Polyclonal antibodies raised in rabbits against bacterially produced peptides in the C-terminal region of *v-erb* A or human *c-erb* A_α oncogenes recognize the nuclear triiodothyronine (T3) receptors in the T3-sensitive Ob 17 mouse preadipocyte cell line and not in mouse or rat liver. The results confirm the existence of different T3 receptors in different tissues. The results also suggest a heterogeneous receptor distribution within the preadipose cell line, with a predominance of c-erb A_α-type species. Antibodies raised against domain 149–227, but not against domain 245–325, impair T3 binding, suggesting a role for this domain in ligand binding.

Thyroid hormone; Receptor; Oncogene, *erb* A; Antibody; (Liver, Preadipocyte)

1. INTRODUCTION

The multiple developmental and metabolic actions of thyroid hormones are thought to be initiated by the binding of 3,5,3′-triiodothyronine (T3) to chromatin-associated receptors [1–3]. The nuclear T3 receptors which were only partially purified from rat liver [4–6] were described in different tissues as one class of high-affinity sites for T3 in saturation analyses [7] and as an apparently homogeneous population in studies of the molecular mass under non-denaturing conditions [8,9]. Nevertheless, when studying the T3 receptors in rat liver and mouse preadipocyte cell lines, we noticed slight but constant differences in the Sephadex G-100 gel filtration behavior [10]. Furthermore, other observations progressively suggested that distinct T3 receptors could exist: (i) at least two molecular species of about 47 kDa and 57 kDa could be seen in SDS-polyacrylamide gel electrophoreses after affinity labeling of the T3 receptors in GH1 cells [11] and rat liver [12]; (ii) slight differences were reported in the relative affinity for T3 analogs in different tissues [13]; (iii) in studies of the receptor down-regulation by T3, only approx. half of the receptor sites were involved in several cell types [14,15]. It was recently suggested that the nuclear T3 receptors are encoded by a gene family the prototype of which is c-erb A_α, the progenitor of the *v-erb* A oncogene of avian erythroblastosis virus [16,17]. This family is in turn part of a large multigene superfamily which comprises steroids, vitamin D3 and retinoic acid receptors [18]. Molecular cloning and sequence analysis of several c-erb A cDNAs have enabled the classification of them into two sub-classes named α and β, the α-type bearing greater similarity to v-erb A [16–24]. Erb A-specific immunosera have been raised in rabbits against protein products corresponding to parts of the 3′-coding region of *v-erb* A or human *c-erb* A_α oncogenes [25]. This prompted us to comparatively reexplore the nuclear T3 receptors in rat and mouse livers and in the mouse preadipocyte cell line Ob 17.

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2. MATERIALS AND METHODS

2.1. *Nuclear extracts; nuclear T3 receptor site estimation*

Ob 17 preadipocytes [26] were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and collected for nuclei purification 3–5 days after confluence without hormone addition [10]. Liver nuclei were purified from male Sprague-Dawley rats (200–250 g) and Swiss mice (20 g) [8]. Over 80% of the nuclear T3 receptor sites were solubilized with 0.4 M KCl in buffer 1 (20 mM Tris-HCl, 1 mM $MgCl_2$, 2 mM EDTA, pH 7.9) (1 ml/300–500 μg DNA). T3-binding-site concentrations and apparent affinity constants K_a were determined in saturation experiments with ^{125}I-T3 (1.5 μCi/μg, Amersham, England) [27]. In analytical fractionations and immunoanalyses, the T3 receptors were either detected through specifically bound ^{125}I-T3 when using nuclear extracts preincubated with 0.3 nM ^{125}I-T3 (16 h, 0°C), or estimated by the ability of the fractions to bind ^{125}I-T3 at a near saturating concentration (1 nM) [15]. Non-specific binding was determined with a 500-fold excess of unlabeled T3 and subtracted. Bound and free ^{125}I-T3 were separated by use of a Dowex 1 × 8 exchange resin which binds free T3 (resin test) [27].

2.2. *Analytical fractionations*

Gel filtrations were performed on Biogel A 0.5m (100–200 mesh) columns (10 × 500 mm) in buffer 1 containing 0.1 M KCl, 10 mM $NaPO_4$, pH 7.9, and 5% glycerol. Sucrose gradient centrifugations were run in 13 ml of 5–20% sucrose gradients in buffer 1 containing 0.4 M KCl, for 18 h at 2°C and 40000 rpm (SW41 rotor). For centrifugations in a denser medium [11], 4 ml of 5–20% sucrose gradients were made in buffer 1 containing 9 vols D_2O/1 vol. H_2O, 0.4 M KCl, 10 mM DTT, 10% glycerol, and run for 40 h at 4°C and 57000 rpm in an SW65 rotor. All analyses were carried out immediately after nuclei extraction.

2.3. *Antibodies to erb A-encoded proteins and immunoanalyses*

Anti-erb A immune rabbit sera (IRS) were raised using peptides produced in *E. coli* from DNA fragments derived from the 3′-coding region of either *v-erb* A or the human (h) *c-erb* A_α oncogenes, as previously described [25]. These peptides cover the following amino acid sequences (chicken c-erb A_α coordinates [16]): 149–227 (bp $22^{MS2\text{-}erbA}$), 228–408 (bp $31^{MS2\text{-}erbA}$) in v-erb A and 245–325 (bp $18^{MS2\text{-}erbA}$) in h c-erb A_α. Antisera 27 (IRS 27) and 28 (IRS 28) were derived from two different rabbits immunized with bp $22^{MS2\text{-}erbA}$; antisera 23 (IRS 23) and 21 (IRS 21) were from two rabbits immunized with bp $31^{MS2\text{-}erbA}$ and bp $18^{MS2\text{-}erbA}$, respectively [25]. The immunoanalyses were carried out by incubating the nuclear extracts with various IRS amounts under conditions of fixed rabbit serum concentration (1:3 (v/v); IRS ± normal rabbit serum (NRS)) and fixed T3 receptor site concentration (200 pM). After 16 h at 0°C, the T3 receptors or pre-formed ^{125}I-T3-receptor complexes were analyzed for: (i) level estimation; (ii) behavior in gel filtration and sucrose gradient centrifugation; (iii) ability to be taken up by sufficient amounts of insoluble protein A in *Staphylococcus aureus* cells (pansorbin, Calbiochem) which did not significantly bind unreacted T3 receptors.

3. RESULTS

In comparative analyses, nuclear extracts from rat or mouse livers, and from Ob 17 mouse preadipocytes, bound T3 to one class of sites with similar high affinities (K_a 1 to 3 × 10^{10} M^{-1}). Biogel A 0.5m gel filtrations of the same nuclear extracts preincubated with ^{125}I-T3 reproduced findings previously obtained when using Sephadex G-100 [8,10]: specifically bound T3 was recovered almost exclusively in an included peak eluting later with preadipocytes as compared to liver (K_{av} 0.50 and 0.38, respectively) (figs 1A,2). Furthermore, the preadipocyte receptor peak was always found to be larger with at least one shoulder at approx. 0.38 K_{av}, and evoked a heterogeneity of the T3 receptor population. After sucrose gradient centrifugations in H_2O, the livers and preadipocyte receptors sedimented at 3.6 S without any significant difference; but, under conditions of sucrose gradient centrifugation in a denser medium, slight differences were constantly reproduced: the liver receptors were found in an apparently homogeneous peak whereas the preadipocyte receptors were recovered in a slightly slower sedimenting peak presenting a shoulder in the position of liver receptors (fig.1B). This suggested that, under non-denaturing conditions, different molecular species of T3 receptors could exist that are differently distributed between tissues.

To analyze further a possible heterogeneity of the T3 receptors, we looked for a reactivity to erb A-specific antisera raised in rabbits against different peptides corresponding to the C-terminal domain of hc-erb A_α (hormone-binding domain) and the corresponding domain of v-erb A [25]. In a first series of assays, nuclear extracts from rat or mouse livers, and from preadipocytes, were incubated with ^{125}I-T3 and then with NRS or the various IRS (under conditions of fixed T3, T3-binding sites and serum concentrations) and analyzed in gel filtration. As shown in fig.2, incubation of these extracts with NRS did not change the T3 receptor elution profiles obtained with untreated extracts (see fig.1A). Similarly, the liver receptor profiles were not significantly changed by IRS 27 and IRS 21. However, both IRS provoked significant and different changes in the preadipocyte receptor profiles. While IRS 27 provoked a decline of the included peak of bound T3 with a

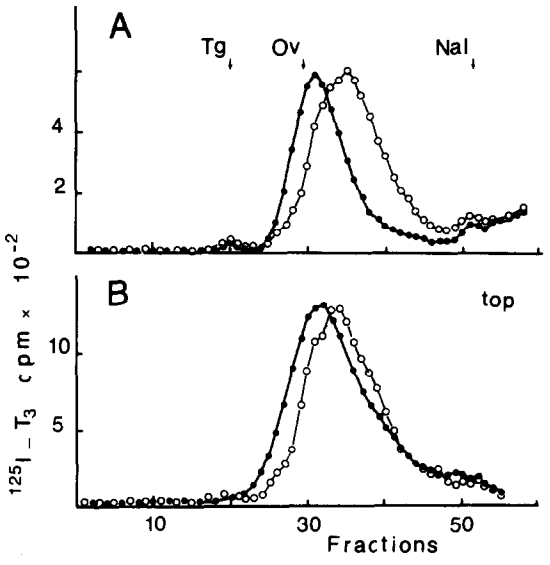


Fig.1. Biogel A 0.5m gel filtration (A) and sucrose gradient centrifugation in the presence of D_2O (B) of rat liver (●) and Ob 17 preadipocytes (○) nuclear extracts. Incubation with ^{125}I-T3 was performed before gel filtration in A and in each fraction in B. The data depict specifically bound T3. The position of thyroglobulin (Tg), ovalbumin (Ov) and NaI in gel filtration is given by the arrows.

parallel increase of the free T3 peak (K_{av} approx. 1.5), IRS 21 partly shifted bound T3 into an excluded peak. Neither NRS nor IRS bound ^{125}I-T3 to a significant level in the absence of nuclear extracts (fig.2, dashed lines in left panels).

The dissociation of preformed ^{125}I-T3-receptor complexes by IRS 27 in preadipocyte nuclear extracts was further evidenced and explored in resin tests. As shown in fig.3, the dissociation was concentration-dependent and virtually complete at 10 μl IRS 27 per fmol T3-binding site. An essentially complete dissociation was also obtained with IRS 28, a serum directed against the same domain as that used to generate IRS 27, at the same relative concentration or with an IRS 28-derived fraction highly enriched in immunoglobulins. By contrast, both IRS only poorly dissociated the complexes formed in rat or mouse liver nuclear extracts. Taken together, these results strongly suggest an authentic antibody effect. Furthermore, a concentration-dependent depletion of T3-binding sites was also observed in preadipocyte nuclear extracts preincubated with IRS 27 and then cleared of the IgGs by using sufficient amounts of pansorbin.

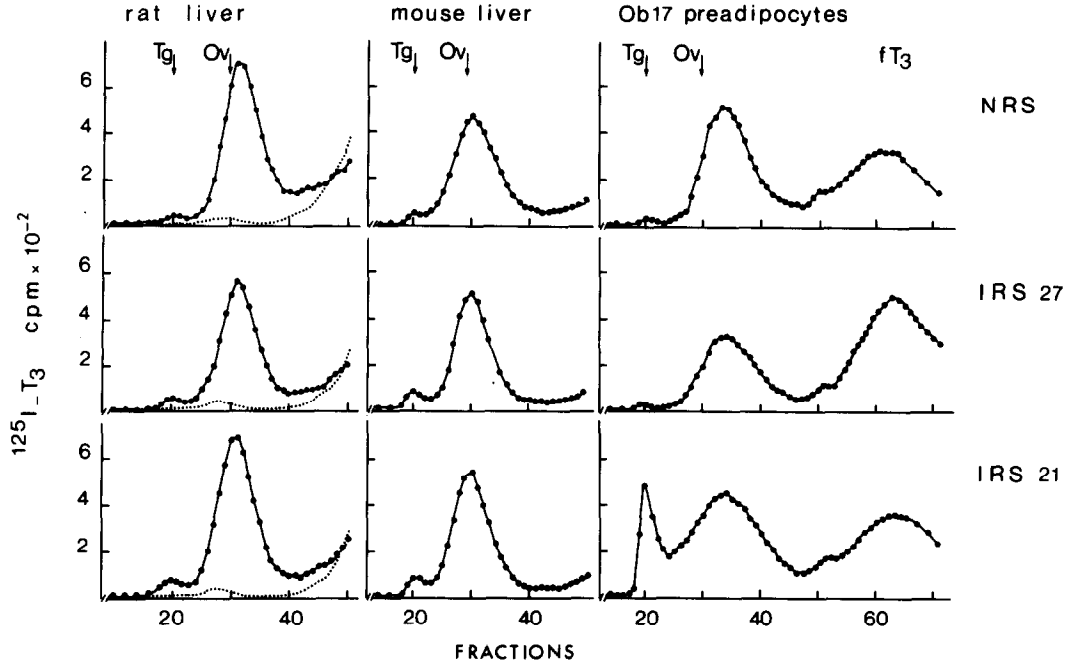


Fig.2. Biogel A 0.5m gel filtration elution profiles of nuclear ^{125}I-T3-receptor complexes of rat or mouse livers and of Ob 17 preadipocytes after incubation with rabbit serum either normal (NRS) or immune (IRS 27 and 21). The elution of free T3 is shown in the righthand panels. The dashed lines in the lefthand panels illustrate the profiles obtained when incubating the respective NRS or IRS with ^{125}I-T3 in the absence of nuclear extract.

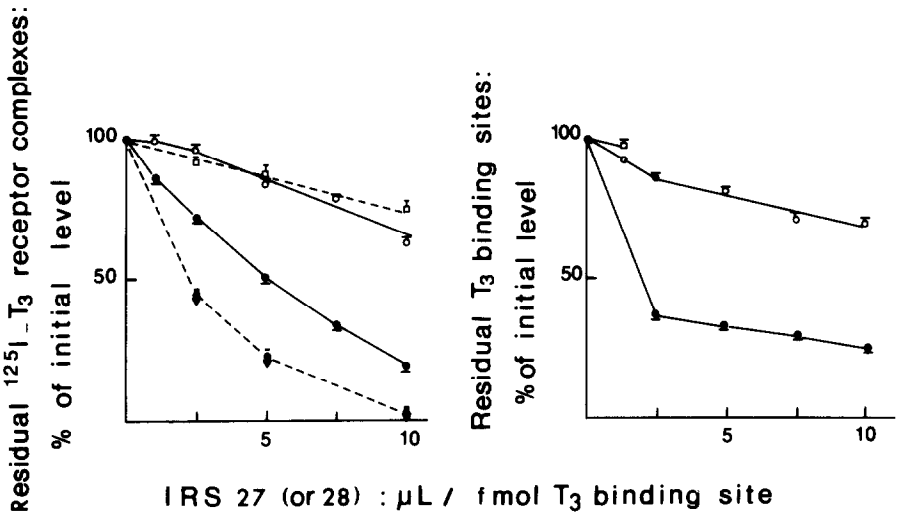


Fig.3. Effect of increasing concentrations of IRS 27 (——) or IRS 28 (- - -) added to nuclear extracts of rat liver (○), mouse liver (□) or Ob 17 preadipocytes (●) on (left) preformed ^{125}I-T3-receptor complex level, estimated in resin tests, (right) residual T3-binding site level estimated after stripping the IgGs with sufficient amounts of insoluble protein A (pansorbin). An immunoglobulin-enriched fraction of IRS 28, reconstituted to the initial volume of serum after two successive salting-outs with $(NH_4)_2SO_4$ (40% saturation) and extensive dialysis with buffer 1, was comparatively analyzed with preadipocytes nuclear extract (▼). The data are means ± SE of triplicate assays.

This receptor depletion occurred in the same range of IRS concentration as described above for T3-receptor dissociation. The liver receptors were poorly affected (fig.3, right).

In contrast to the results obtained using IRS 27 and IRS 28, IRS 21 never dissociated preformed ^{125}I-T3-receptor complexes (or impaired T3 binding) in either nuclear extract. However, a concentration-dependent shift of T3-receptor complexes from the main included peak, in gel filtration, to an excluded one was always observed with the preadipocyte receptors (fig.4). A shift of bound T3 to faster sedimenting species was also observed in sucrose gradient centrifugation (not shown). Quantitatively, the amount of bound ^{125}I-T3 shifted to higher M_r species roughly paralleled the amount which was found to be retained by pansorbin in immunoprecipitation analyses. These data are collected in fig.5. Also apparent in fig.5 is the absence of reaction of IRS 21 with liver receptors and the fact that, in preadipocytes, only part of the T3-receptor complex population seems to be involved in the immune recognition. When preincubating the nuclear extracts with IRS 21, then pansorbin, the T3-binding sites measured in a subsequent incubation of the supernatants with ^{125}I-T3 declined in a similar IRS concentration-dependent fashion. Fig.4 also suggests that the T3-receptor complexes in preadipocyte nuclear extracts are not uniformly recognized by the IRS 21 antibodies since the shift appears to affect preferentially the T3-receptor species which elute at K_{av} above 0.38.

A third type of immune serum, IRS 23, also ex-

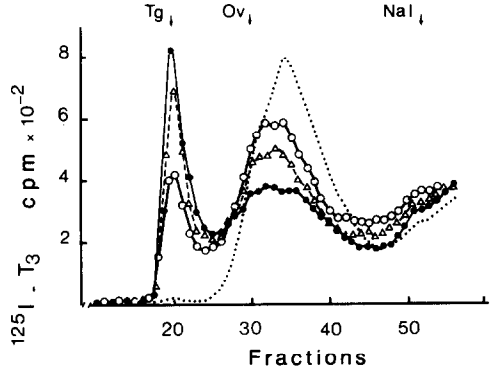


Fig.4. Effect of increasing concentrations of IRS 21 on the gel filtration behavior (Biogel A 0.5m) of preadipocytes ^{125}I-T3-receptor complexes: 0 (···), 1.5 (○—○), 3.0 (Δ---Δ) and 5.0 (●—●) µl per fmol T3-binding site.

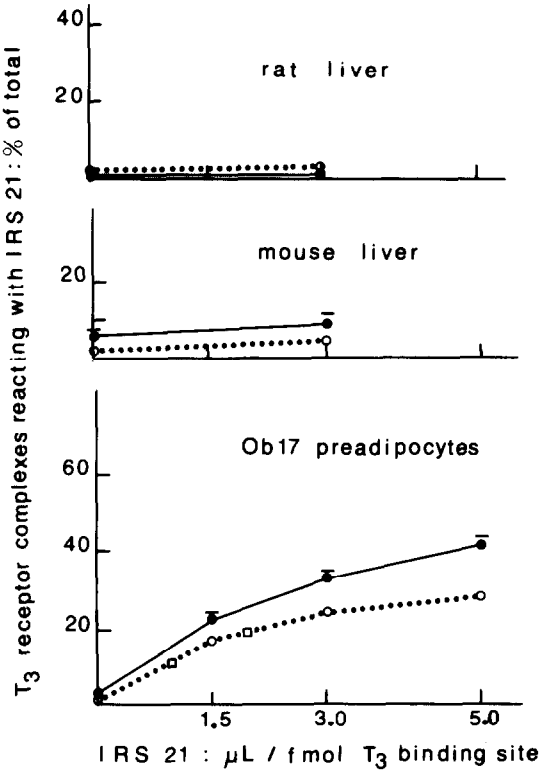


Fig.5. Effect of increasing concentrations of IRS 21 on the fraction of preadipocytes ^{125}I-T3-receptor complexes shifted to the excluded peak in gel filtration (□,○ separate experiments) or retained by insoluble protein A (●).

clusively recognized the preadipocyte receptors and presented mixed characteristics: partial inhibition of T3 binding (and dissociation of T3-receptor complexes), and a shift of bound T3 to excluded species in Biogel A 0.5m gel filtration (not shown).

4. DISCUSSION

The present study demonstrates, for the first time, that polyclonal antibodies directed against different peptide portions of the C-terminal part of v-erb A or hc-erb A_α encoded proteins can recognize nuclear T3 receptors present in mouse preadipose cells. The same antibodies did not significantly recognize the receptors present in rat or mouse liver, as also recently reported [28]. The antibodies we used in this study are able to recognize the protein products encoded by the c-erb A_α gene family, whereas only one of them (IRS 23) recognizes the products of both α- and β-type c-erb A genes ([25,28] and unpublished results). Our results therefore favor the hypothesis that most of the T3 receptors of mouse preadipocytes belong to the α-family. Consistent with recently published results [24,28] liver receptors mostly belong to the β-subtype.

Our results demonstrate that nuclear T3 receptors, as defined by in vivo-formed high-affinity T3-binding entities, are differently distributed in different tissues. These results also suggest that, within a single-cell type, a preadipocyte cell line, a heterogeneous expression of T3 receptors might exist. The presence of T3 binding molecular species generated by a partial degradation of the receptor cannot be totally excluded. Nevertheless, the existence of different molecular forms of T3 receptors can also be evoked. Whether this implies the coexistence of T3 receptors of the α- and β-subfamilies of *c-erb* A oncogenes or the existence of several α-type isoforms is under investigation at the protein and mRNA levels. Furthermore, we reported an increase of the T3 receptor concentration during adipose differentiation [10]; a possible evolution of the T3 receptor population has thus to be evaluated.

Interestingly, we found different patterns of interaction when analyzing the preadipocyte T3 receptors with the different antisera. IRS 21 antibodies recognized the preadipocyte receptors without impairing T3 binding. Thus, the corresponding epitopes in the amino acid sequence 245–325 of hc-erb A_α are not significantly involved in the constitution of the hormone binding site. IRS 27-28 and IRS 23 antibodies correspond to two successive sequences of v-erb A protein covering a large part of the C-terminal region following the DNA-binding domain [25]. The IRS 23 immunogen (bp $31^{MS2\text{-}erbA}$) comprises a sequence of large homology with that of the IRS 21 immunogen (bp $22^{MS2\text{-}erbA}$) (only two amino acid changes). Both types of antisera presumably recognize some common epitope(s) involved in receptor recognition without impairing T3 binding. However the IRS 23 immunogen also generated antibodies which impaired T3 binding, an event exclusively observed with IRS 27-28 antibodies. The in vitro-expressed v-erb A protein was found to be unable to bind T3 [16]; but our results suggest that it contains important sequences, in distinct regions, for

the constitution of a stabilized conformation of the T3-binding site, at least in the α-type receptors. It is worth noting that a recognition of the liver receptors by IRS 27-28 antibodies could not be totally excluded: the slight inhibition of T3 binding that we observed in their presence could be related to either a low affinity in the liver receptors/antibodies interaction or to the presence of other erb A protein species which do not bind T3 as recently reported [23] and which compete with the receptors for antibody recognition. A knowledge of the T3-binding site in physiologically expressed T3 receptors will be gained from mutation-deletion experiments as recently reported [29] but also needs the development of monospecific and monoclonal antibodies to T3 receptor/*erb* A oncogene products.

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REFERENCES

[1] Menezes-Ferreira, M.M. and Torresani, J. (1983) Ann. Endocrinol. 44, 205–216.
[2] Oppenheimer, J.H., Schwartz, H.L., Mariash, C.N., Kinlaw, W.B., Wong, N.C.W. and Freake, H.C. (1987) Endocrinol. Rev. 8, 288–308.
[3] Samuels, H.H., Forman, B.M., Horowitz, Z.D. and Ye, Z.S. (1988) J. Clin. Invest. 81, 957–967.
[4] Silva, E.S., Astier, H., Thakare, V., Schwartz, H.L. and Oppenheimer, J.H. (1977) J. Biol. Chem. 252, 6799–6805.
[5] Torresani, J. and Anselmet, A. (1978) Biochem. Biophys. Res. Commun. 81, 147–153.
[6] Ichikawa, K. and DeGroot, L.J. (1987) Proc. Natl. Acad. Sci. USA 84, 3420–3424.
[7] Oppenheimer, J.H., Koerner, D., Schwartz, H.L. and Surks, M.I. (1972) Endocrinology 95, 897–903.
[8] Torresani, J., Anselmet, A. and Wahl, R. (1978) Mol. Cell. Endocrinol. 9, 321–333.
[9] Perlman, A.J., Stanley, F. and Samuels, H.H. (1982) J. Biol. Chem. 257, 930–938.
[10] Anselmet, A., Gharbi-Chihi, J. and Torresani, J. (1984) Endocrinology 114, 450–455.
[11] Casanova, J., Horowitz, Z.D., Copp, R.P., McIntyre, W.R., Pascual, A. and Samuels, H.H. (1984) J. Biol. Chem. 259, 12084–12091.
[12] Dozin, B., Cahnmann, H.J. and Nikodem, V. (1985) Biochemistry 24, 5197–5202.
[13] Underwood, A.H., Emmett, J.C., Ellis, D., Flynn, S.B., Leeson, P.D., Benson, G.M., Novelli, R., Pearce, N.J. and Shah, V.P. (1986) Nature 324, 425–429.
[14] Samuels, H.H., Stanley, F. and Shapiro, L.E. (1977) J. Biol. Chem. 252, 6052–6060.
[15] Pou, M.A., Bismuth, J., Gharbi-Chihi, J. and Torresani, J. (1986) Endocrinology 119, 2360–2367.
[16] Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H. and Vennström, B. (1986) Nature 324, 635–640.
[17] Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J. and Evans, R.M. (1986) Nature 324, 641–646.
[18] Evans, R.M. (1988) Science 240, 889–895.
[19] Benbrook, D. and Pfahl, M. (1987) Science 238, 788–791.
[20] Thompson, C.C., Weinberger, C., Lebo, R. and Evans, R.M. (1987) Science 237, 1610–1614.
[21] Izumo, S. and Mahdavi, V. (1988) Nature 334, 539–542.
[22] Nakai, A., Seino, S., Sakurai, A., Szilak, I., Bell, G.I. and DeGroot, L.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2781–2785.
[23] Mitsuhashi, T., Tennyson, G.E. and Nikodem, V. (1988) Proc. Natl. Acad. Sci. USA 85, 5804–5808.
[24] Murray, M.B., Zilz, N.D., McCreary, N.L., MacDonald, M.J. and Towle, H.C. (1988) J. Biol. Chem. 263, 12770–12777.
[25] Goldberg, Y., Glineur, C., Gesquiere, J.C., Riconart, A., Sap, J., Vennström, B. and Ghisdael, J. (1988) EMBO J. 7, 2425–2433.
[26] Negrel, R., Grimaldi, P. and Ailhaud, G. (1978) Proc. Natl. Acad. Sci. USA 75, 6054–6058.
[27] Torresani, J. and DeGroot, J.J. (1975) Endocrinology 96, 1201–1209.
[28] Freake, H.C., Santos, A., Goldberg, Y., Ghysdael, J. and Oppenheimer, J.H. (1988) Mol. Endocrinol. 2, 986–991.
[29] Munoz, A., Zenke, M., Gehring, U., Sap, J., Beug, H. and Vennström, B. (1988) EMBO J. 7, 155–159.