Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 19, Issue of May 10, pp. 11055–11058, 1996

Induction of Calmodulin Kinase IV by the Thyroid Hormone during the Development of Rat Brain*

(Received for publication, February 22, 1996)

Joachim Krebs‡§, Raylene L. Means¶, and Paul Honegger‖

From the ‡Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland, the ¶Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, and the ‖Institute of Physiology, University of Lausanne, CH-1005 Lausanne, Switzerland

This communication reports the specific induction of calmodulin kinase IV by the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) in a time- and concentration-dependent manner at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system, which can grow and differentiate under chemically defined conditions. The induction of the enzyme that can be observed both on the mRNA and on the protein level is T_3-specific, *i.e.* it cannot be induced by retinoic acid or reverse T_3, and can be inhibited on both the transcriptional and the translational level by adding to the culture medium actinomycin D or cycloheximide, respectively. The earliest detection of calmodulin kinase IV in the fetal brain tissue of the rat is at days E16/E17, both on the mRNA as well as on the protein level. This is the first report in which a second messenger-dependent kinase involved in the control of cell regulatory processes is itself controlled by a primary messenger, the thyroid hormone.

As one of the intracellular second messengers, calcium plays a central role in cell growth and differentiation (1). The primary receptor protein mediating the calcium signal inside the cell is calmodulin (CaM),[1] which regulates, among others, protein kinases and protein phosphatase(s). Next to the well studied multifunctional CaMKII (see Ref. 2 for a recent review), CaMKIV recently received considerable attention. In contrast to the ubiquitous CaMKII, CaMKIV is more restricted in its expression in different tissues. The highest levels of the enzyme in mammalian tissues can be found in brain, thymus, and, to a somewhat lower extent, in testis and spleen, whereas in other tissues the enzyme remained undetectable (3–7). The amino acid sequence of CaMKIV, which exists in two monomeric isoforms of M_r 65,000 (α) and M_r 67,000 (β) due to alternative splicing, has been deduced from rat, mouse, and human brain cDNAs (4, 5, 8–10), demonstrating less than 50% homology to the corresponding regions of CaMKII. Next to the rather poor sequence homology between the two CaM kinases, CaMKII and IV, the two enzymes also seem to differ in their activation mechanism. Whereas CaMKII can be efficiently activated by autophosphorylation (2), the activation of CaMKIV by autophosphorylation is rather slow and inefficient (11, 12). Recent reports seem to indicate that CaMKIV is activated by a CaMKIV kinase (13–16), reminiscent of the regulation of mitogen-activated protein kinase activity by a kinase cascade.

In comparison to CaMKII, the substrate specificity of CaMKIV seems to be more restricted. Apart from synapsin I, which is a substrate for both CaM kinases, the only other substrates reported for CaMKIV are the Ras-related GTP-binding protein Rap-1b (17), the cAMP regulatory element-binding protein CREB (11, 18), the serum response factor SRF (19), and members of the Ets family of transcription factors (20, 21). The recent report of a substantial localization of CaMKIV in the nucleus (18, 22) permits the enzyme direct access to these transcription factors to regulate their function in a Ca^{2+}-dependent manner. Thus, it has been reported that CaMKIV is involved in the Ca^{2+}-dependent regulation of expression of immediate early genes either through CREB (Refs. 12, 18, 23, and 24; see also Ref. 25) or through SRF (19).

Since thyroid hormones have been shown to be required for normal growth and differentiation of the mammalian brain (26–28), we initiated studies to investigate the influence of the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) on the expression of a number of neuronal and glial membrane markers during development in rat brain cell cultures (29–31). Here we report the specific induction of CaMKIV by T_3 in a time- and concentration-dependent manner at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system, which can grow and differentiate under chemically defined conditions (32). The induction is T_3-specific, *i.e.* the expression of the enzyme cannot be induced by either reverse T_3 or retinoic acid. The expression of CaMKIV is regulated on both the transcriptional and the translational level, since both the addition of actinomycin D as well as cycloheximide to the cultural medium can prevent the T_3-dependent induction of the enzyme. In addition, the T_3-specific induction can be observed both on the mRNA and on the protein level. This is the first report in which a second messenger-dependent kinase involved in the control of cell regulatory processes is itself controlled by a primary messenger, the thyroid hormone. Preliminary accounts of part of the data presented here have been given elsewhere (33, 34).

EXPERIMENTAL PROCEDURES

Cell Culture—Serum-free, rotation-mediated aggregating cell cultures were prepared from fetal (15 days of gestation) rat (OFA/Ico/Ibm strain, Biological Research Laboratories Ltd., Basel, Switzerland) telencephalon, as described in detail previously (32). Aliquots of 6×10^7 cells were transferred to DeLong flasks and maintained under constant gyratory agitation at 37 °C, in an atmosphere of 10% CO_2 and 90% humidified air. The culture medium used was Dulbecco's modified Eagle's medium (Life Technologies, Inc.), supplemented with nutritional factors, vitamins, trace elements, transferrin (1 mg/liter), insulin (800 nM), and hydrocortisone 21-phosphate (20 nM). Gentamicin sulfate (25

* This work was supported by the Swiss National Science Foundation Grants 31-30858.91 and 31-37292.93 (to J. K. and P. H.), and by a National Institutes of Health Grant HD-07503 (to A. R. Means). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 41-1-632-3005; Fax: 41-1-632-1213.

[1] The abbreviations used are: CaM, calmodulin; CaMKII, Ca^{2+}-CaM-dependent protein kinase II; CaMKIV, Ca^{2+}-CaM-dependent protein kinase IV or Gr; CREB, cAMP response element-binding protein; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SRF, serum response factor; T_3, 3,3′,5-triiodo-L-thyronine; PBS, phosphate-buffered saline.

mg/liter) was used as an antibiotic. For analyses, the aggregates of each flask were washed twice with 5 ml of phosphate-buffered saline (PBS).

Preparation of Cell Extracts—Proteins were extracted, usually from the pellet of whole aggregates (4–5 × 10^7 cells), by adding 200 μl of 25 mM HEPES, pH 7.5, 5 mM EGTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptine, 1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1,10-phenanthroline, 1 mM *p*-aminobenzamidine (buffer A), and incubated on ice for 10 min after extensive vortexing. After centrifugation at 25,000 × *g* for 5 min, proteins in the supernatant were stored at −70 °C.

SDS-PAGE and Electrophoretic Blotting—To 40 μl of supernatants of cellular extracts, 20 μl of loading buffer (35) was added and the proteins were separated on a 10% SDS-PAGE (35). After electrophoretic transfer onto nitrocellulose sheets for 2 h using 240 mM glycine, 30 mM Tris, 20% methanol, and 0.02% SDS as transfer buffer, the blots were incubated overnight at 4 °C with 2% milk powder in PBS as blocking buffer, afterward incubated at room temperature for 3 h with ^{125}I-CaM (36) (2 × 10^5 cpm/ml) in 2% milk powder/PBS containing either 1 mM $CaCl_2$ or 5 mM EGTA, washed once with 2% milk powder/PBS containing either $CaCl_2$ or EGTA, then with PBS containing either $CaCl_2$ or EGTA, and third with PBS. Each washing was done for 15 min. After drying the blots under cold air, the sheets were exposed either to x-ray sensitive films (Kodak XAR-5) at −70 °C or to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA) at room temperature and developed. Quantitation was obtained using the PhosphorImager software as recommended by the manufacturers. CaM was purified from bovine brain as described previously (37).

Preparation and Characterization of Total RNA—For RNA preparation, cell culture aggregates (6–8 × 10^8 cells) were grown for 5 days either in the absence or in the presence of 3 × 10^{-8} M T_3, washed three times with PBS, and quickly frozen in liquid nitrogen. Total RNA was isolated by using the Ultraspec RNA kit (Biotecx Laboratories, Inc., Houston, TX) as described by the manufacturer. For the identification of CaMKIV-specific mRNA, 40 μg of total RNA was denatured and size-fractionated on a 1.5% agarose, 2.2 M formaldehyde gel. Electrophoresis was carried out at 30 V for 16 h with circulating Na_3PO_4 buffer at 4 °C. Prior to blotting the gels were stained with ethidium bromide to identify 18 and 28 S rRNA as markers. The RNA was transferred onto Biotrans nylon membranes (ICN Biomedicals, Inc., Costa Mesa, CA) and stabilized by using UV cross-linking. Northern blots were prehybridized and hybridized at 37 °C in 50% formamide as described previously (38). The probe was a 1-kilobase pair (*Bam*HI-*Kpn*I) fragment of the rat CaMKIV cDNA (4) and was labeled with [α-^{32}P]dCTP according to the random oligonucleotide priming method of Feinberg and Vogelstein (39).

Purification and Characterization of p64—Aggregates of a total of 20 culture flasks (~10^9 cells), grown for 5 days in the presence of T_3 (3 × 10^{-8} M), have been homogenized at 4 °C in three volumes of ice-cold buffer A, basically following the purification protocol for CaMKIV described in Ref. 40. Briefly, the lysates were centrifuged at 100,000 × *g* for 30 min, and the supernatants were pooled and applied to a 15-ml DEAE-cellulose column equilibrated with 25 mM Hepes, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT (buffer B). The column was washed with 10 bed volumes of buffer B, and bound proteins were eluted with a stepwise gradient using buffer B and increments of 100 mM NaCl at each step. p64 eluted between 150 and 300 mM NaCl. The fractions containing p64 were pooled and applied onto a prepacked 1-ml hydroxylapatite column from Bio-Rad equilibrated with buffer B containing 200 mM NaCl (buffer C). The column was washed with five bed volumes of buffer C, and bound proteins were eluted with a linear 0–200 mM NaH_2PO_4 gradient. p64-containing fractions eluting between 75 and 100 mM NaH_2PO_4 were pooled, dialyzed overnight against 5 liters of 25 mM Hepes, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT (buffer D), and applied to a 5-ml CaM column (36) equilibrated with buffer D. After washing the column with 10 bed volumes of buffer D containing 10% glycerol (buffer E), followed by a wash of five bed volumes with buffer D containing 0.5 M NaCl, p64 was eluted by a buffer containing 25 mM Hepes, pH 7.5, 2 mM EDTA, 5 mM EGTA, 1 mM DTT, 10% glycerol. Purified p64 was identified as a calmodulin-dependent kinase by autophosphorylation using the assay as described by Cruzalegui and Means (11). Autophosphorylated CaMKII α and β were identified by specific antibodies, kindly provided by Dr. H. Schulman. Identification of p64 as CaMKIV by specific antibodies was carried out by Western blotting using 2% milk powder/PBS as blocking buffer.

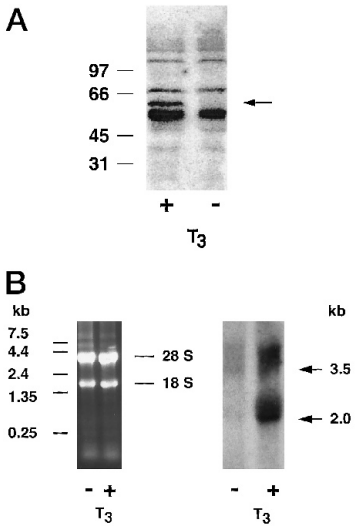

FIG. 1. **Identification of CaMKIV in fetal rat telencephalon cell culture extracts grown in the presence or absence of T_3.** Soluble proteins (*A*) or total RNA (*B*) extracted from cultures grown for 5 days in the presence (+) or absence (−) of 3 × 10^{-8} M T_3 were separated on either SDS-PAGE (*A*) or agarose gels (*B*), and after electroblotting onto appropriate membranes identified by either ^{125}I-CaM (*A*) or by a CaMKIV-specific cDNA probe (*B*), as described in detail under "Experimental Procedures." Molecular size standards (in kDa) are indicated at the *left* of *panel A*; the *arrow* denotes the location of CaMKIV (p64). The *numbers* on the *right* of *panel B* (*right panel*) indicate the calculated sizes of the hybridizing bands. Migration of nucleic acid size standards are shown at the *left* of *panel B* (*left panel*), which shows the ethidium bromide staining of the same gel prior to blotting. *28S* and *18S* mark the levels of the corresponding ribosomal RNA species.

RESULTS AND DISCUSSION

Aggregating cell cultures prepared from 15-day fetal rat telencephalon and grown for 5 days in the presence or absence of the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) in a chemically defined medium were collected and extracted with an EGTA-containing buffer. The extracted proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose, and CaM-binding proteins were identified by incubation with ^{125}I-labeled CaM in the presence or absence of Ca^{2+}. As shown in Fig. 1*A*, a number of different CaM-binding proteins were present in the M_r range between 40,000 and 160,000, which could not be detected in the presence of EGTA (data not shown). By comparing cultures that were grown for 5 days either in the presence or absence of 3 × 10^{-8} M T_3, it was obvious that one CaM-binding protein with a M_r of 64,000 (later identified as CaMKIV; see below) appeared only in cultures grown in the presence of T_3, suggesting that its expression depended on this hormone. This result could be corroborated by the observation that in Northern blots of total RNA isolated from similar cell cultures a mRNA of 2 kilobases could be clearly identified by a probe specific for CaMKIV (Fig. 1*B*). This band could only be observed in the presence of T_3, but not in its absence (even at higher RNA concentrations; data not shown). On the other hand, a band of 3.5 kilobases, which has been described before as a much less abundant mRNA of CaMKIV in adult rat brain (3, 4), could be observed as a weak band even in the absence of T_3-induced cultures. The difference between the two mRNAs could derive from differences in the 3′-untranslated region by use of alternate poly(A) sites as discussed before (4), but the reason for the apparent difference in the T_3-dependent expression between the two mRNAs needs further investigation.

Since the apparent molecular weight and autophosphorylation kinetics showed similarities between p64 and CaMKIV we attempted to purify p64 from a pool of aggregate cultures

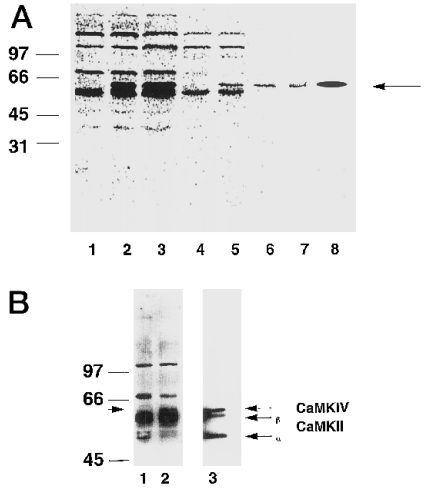

FIG. 2. **Purification of p64 and identification as CaMKIV by specific antibodies (*A*) and by autophosphorylation (*B*).** *A*, protein extracts of fetal rat telencephalon cell cultures grown in the absence (*lane 1*) or presence (*lane 2*) of 3×10^{-8} M T_3 were separated by SDS-PAGE, blotted onto nitrocellulose, and incubated with ^{125}I-CaM as described under "Experimental Procedures." *Lane 3*, aliquot of the starting material for p64 purification. *Lanes 4–6*, fractions from the DEAE-cellulose eluate containing 100 mM NaCl (*lane 4*), 200 mM NaCl (*lane 5*), and 300 mM NaCl (*lane 6*). p64 was purified to homogeneity by hydroxylapatite column and CaM affinity chromatography (*lane 7*) and identified as CaMKIV by specific antibodies (*lane 8*). *B*, identification of p64 as a CaM kinase by autophosphorylation. Purified p64 and CaMKII α and β were autophosphorylated by using [^{32}P-γ]ATP in a Ca^{2+}/CaM-dependent manner as described in Ref. 11 and separated by SDS-PAGE (*lane 3*). Identification of p64 in fetal rat telencephalon cell cultures grown in the absence (*lane 1*) or presence (*lane 2*) of 3×10^{-8} M T_3. p64 is indicated by the *arrow*. Molecular size standards (in kDa) are indicated at the *left* of *panels A* and *B*; CaMKIV and CaMKII α and β are indicated at the *right* of *panel B*.

grown for 5 days in the presence of 3×10^{-8} M T_3 by applying a modification of the purification protocol of Hanissian *et al.* (Ref. 40; see also "Experimental Procedures"). As shown in Fig. 2, p64 eluted from the DEAE-cellulose column at a concentration of NaCl between 200 and 300 mM, typical for CaMKIV. Further purification was obtained by using a hydroxylapatite column, and finally a CaM affinity column. After extensive washing with a calcium-containing buffer, p64 was eluted using an EGTA-containing buffer (Fig. 2). The purified protein was identified as a CaM-dependent kinase by CaM-dependent autophosphorylation (Fig. 2*B*) and as CaMKIV by specific antibodies (Fig. 2*A*). Using immunoprecipitation CaMKIV could be identified only in cells that had been grown in the presence of T_3 (data not shown). With respect to its M_r of 64,000–65,000 and its prenatal appearance, p64 most likely represents the α-isoform of CaMKIV since the β-polypeptide has a slightly higher M_r, *i.e.* 67,000, is specifically expressed in brain only in cerebellum, and can be observed only postnatally (6).

It was noted that autophosphorylation of pure p64 was rather slow, as described by Cruzalegui and Means (11) for a recombinant CaMKIV. On the other hand, slow phosphorylation of p64 could also be indicative for the presence of contaminating amounts of the recently described CaMKIV kinase (13–16). The other CaM-binding proteins exhibiting M_r values between 60,000 and 63,000 were identified as CaMKIIβ and calcineurin A, respectively, by using monoclonal antibodies against CaMKIIβ and by comparison with purified calcineurin. It appeared that in contrast to CaMKIV, the expression of both CaMKII and calcineurin was independent of T_3 (data not shown).

The influence of T_3 was studied further in a dose- and time-dependent expression of CaMKIV. As shown in Fig. 3, the enzyme was detectable already at very low concentrations of T_3 (3×10^{-10} M; Fig. 3) and increased in intensity with increasing

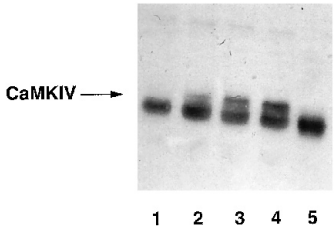

FIG. 3. **^{125}I-CaM overlay of extracts of fetal rat telencephalon cell cultures grown in the absence (*lanes 1* and *5*, control) or in the presence of 3×10^{-10} M T_3 (*lane 2*), 3×10^{-9} M T_3, (*lane 3*), and 3×10^{-8} M T_3 (*lane 4*).** The *arrow* indicates CaMKIV.

concentrations of T_3 in the culture medium, indicating that the induction of CaMKIV by T_3 was dose-dependent. In addition, when cultures received T_3 (3×10^{-8} M) for various lengths of time, CaMKIV was already detectable after 6 h of stimulation (Fig. 4), and the amount of CaMKIV increased as a function of the duration of the stimulus until maximal expression was reached between 24 and 48 h, suggesting that protein synthesis played a role in the induction of this gene. This interpretation was corroborated by incubating the cell cultures with either actinomycin D or cycloheximide, respectively, to prevent transcription or protein synthesis of the inducible gene. As can be seen from Fig. 5, CaMKIV was clearly induced after exposure to 3×10^{-8} M T_3 for 24 h (Fig. 5, *lane 3*; see also Fig. 4, *lane 4*), but the protein was not detectable if the cultures had been preincubated for 1 h with either 1 μM actinomycin D (Fig. 5, *lane 5*) or 5 μM cycloheximide (Fig. 5, *lane 4*), respectively, before T_3 was added. This is in contrast to the other detectable calmodulin-binding proteins, which during the time period of observation (*i.e.* 24 h) were independent of transcription or translation.

A further observation supporting the view that the expression of CaMKIV was a T_3-specific process was the finding that next to T_3 only T_4 was able to induce CaMKIV (Fig. 6, *lanes 2* and *3*). On the other hand, neither reverse T_3 nor retinoic acid could induce the expression of CaMKIV (Fig. 6, *lanes 4* and *5*). In addition, neither nerve growth factor nor epidermal growth factor were able to induce the expression of the kinase (data not shown), although both growth factors have been demonstrated to regulate developmental processes in these cultures (41, 42). Also, CaMKIV may be specifically expressed in neurons, since it was found at high levels in neuron-enriched aggregate cultures (data not shown), in which highly proliferating glial cells have been suppressed by the addition of 1-β-D-arabino-furanosylcytosine (Ara-C) (31).

Recent reports suggested that CaMKIV is responsible for the Ca^{2+}-dependent regulation of expression of a number of immediate early genes such as c-*fos*, due to the phosphorylation of the cAMP-responsive element-binding protein (CREB) (12, 18, 23, 25) or the serum response factor SRF (19). Our results indicate that during rat brain development the expression of CaMKIV, not detectable at the early stages of ontogenesis (*i.e.* at E15; data not shown) is regulated by the thyroid hormone in a time- and concentration-dependent manner. Whether this T_3-dependent regulation is due to a direct interaction of the T_3-receptor with a responsive element of the CaMKIV gene (28) or whether the effect is indirect remains to be determined, but since the T_3-specific induction of CaMKIV could also be observed on the mRNA level, this observation could be indicative for a T_3-receptor-dependent regulation. In this respect it is of interest that in a recent abstract (43), it was reported that in a mouse embryonic stem cell-derived neuronal culture system the expression of CaMKIV was strictly dependent on the presence of the thyroid hormone receptor. In addition, it should be noted that Shakagami *et al.* (7) observed a rather late appear-

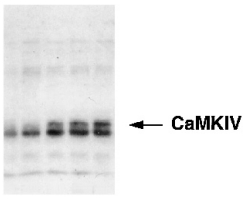

FIG. 4. **^{125}I-CaM overlay of extracts of fetal rat telencephalon cell cultures grown in the absence (*lane 1*) or in the presence of 3×10^{-8}M T_3.** The cell cultures were harvested after 6 h (*lane 2*), 12 h (*lane 3*), 24 h (*lane 4*), or 48 h (*lane 5*). The *arrow* identifies CaMKIV.

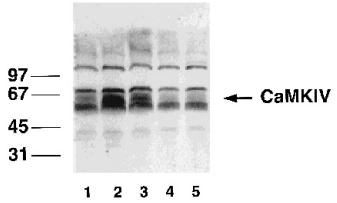

FIG. 5. **^{125}I-CaM overlay of extracts of cell cultures grown in the presence of either cycloheximide or actinomycin D.** The proteins of the cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose as described under "Experimental Procedures." Cell cultures were grown for 5 days (*lanes 1* and *2*) either in the absence (*lane 1*) or in the presence of 3×10^{-8} T_3 (*lane 2*), for 24 h in the presence of 3×10^{-8} T_3 (*lane 3*, control) or preincubated for 1 h with either 5 μM cycloheximide (*lane 4*) or 1 μM actinomycin D (*lane 5*) prior to the incubation with 3×10^{-8} T_3 for 24 h. Molecular size standards (in kDa) are indicated at the *left*; the *arrow* denotes the location of CaMKIV.

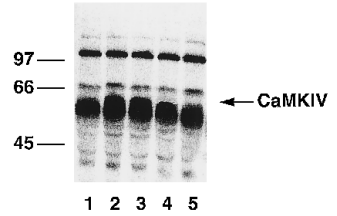

FIG. 6. **^{125}I-CaM overlay of extracts of cell cultures grown in the presence of different hormones.** The proteins of the cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose as described under "Experimental Procedures." Cell cultures were grown for 5 days either in the absence of T_3 (*lane 1*) or in the presence (3×10^{-8} M) of either T_3 (*lane 2*), T_4 (*lane 3*), reverse T_3 (*lane 4*), or retinoic acid (*lane 5*). Molecular size standards (in kDa) are indicated at the *left*; the *arrow* denotes the location of CaMKIV.

ance of the mRNA of CaMKIV during rat embryonal development, between days 15 and 18 of gestation, *i.e.* at about the onset of the embryonal synthesis of the thyroid hormone (44). These findings strongly corroborate our observation that the presence of the thyroid hormone is essential for the expression of CaMKIV. It will be now of interest whether the expression of the CaMKIV kinase is also regulated during brain development and how it correlates with the expression of CaMKIV. In addition, it will be important to know whether in the immune system and in testis CaMKIV is also under the control of the thyroid hormone or whether different signals regulate the expression. Preliminary results indicate that the synthesis of CaMKIV in the rat embryonal thymus is induced following the same time course as in the embryonal brain.[2]

[2] J. Krebs, unpublished observations.

Acknowledgments—We are indebted to Dr. H. Schulman (Stanford University, Stanford, CA) for kindly providing specific antibodies against CaMKII α and β, and to Dr. C. B. Klee (National Institutes of Health, Bethesda, MD) for kindly providing calcineurin. We thank Dr. Peter James for critical reading of the manuscript.

REFERENCES

1. Means, A. R. (1994) *FEBS Lett.* **347,** 1–4
2. Hanson, P. I., and Schulman, H. (1992) *Annu. Rev. Biochem.* **61,** 559–601
3. Ohmstede, C.-A., Jensen, K. F., and Sahyoun, N. E. (1989) *J. Biol. Chem.* **264,** 5866–5875
4. Means, A. R., Cruzalegui, F., LeMagueresse, B., Needleman, D., Slaughter, G., and Ono, T. (1991) *Mol. Cell. Biol.* **11,** 3960–3971
5. Ohmstede, C.-A., Bland, M. M., Merrill, B. M., and Sahyoun, N. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5784–5788
6. Frangakis, M. V., Chatila, T., Wood, E. R., and Sahyoun, N. E. (1991) *J. Biol. Chem.* **266,** 17592–17596
7. Sakagami, H., Watanabe, M., and Kondo, H. (1992) *Mol. Brain Res.* **16,** 20–28
8. Jones, D. A., Glod, J., Wilson-Shaw, D., Hahn, W. E., and Sikela, J. M. (1991) *FEBS Lett.* **289,** 105–109
9. Sakagami, H., and Kondo, H. (1993) *Mol. Brain Res.* **19,** 215–218
10. Bland, M. M., Monroe, R. S., and Ohmstede, C.-A. (1994) *Gene* (*Amst.*) **142,** 191–197
11. Cruzalegui, F. H., and Means, A. R. (1993) *J. Biol. Chem.* **268,** 26171–26178
12. Enslén, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 15520–15527
13. Okuno, S., and Fujisawa, H. (1993) *J. Biochem.* (*Tokyo*) **114,** 167–170
14. Okuno, S., Kitani, T., and Fujisawa, H. (1994) *J. Biochem.* (*Tokyo*) **116,** 923–930
15. Tokumitsu, H., Brickey, D. A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 15520–15527
16. Selbert, M. A., Anderson, K. A., Huang, Q.-H., Goldstein, E. G., Means, A. R., and Edelman, A. M. (1995) *J. Biol. Chem.* **270,** 17616–17621
17. Sahyoun, N. E., McDonald, O. B., Farrell, F., and Lapetina, E. G. (1991) *Proc. Natl. Acad. Sci., U. S. A.* **88,** 2643–2647
18. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol.* **14,** 6107–6116
19. Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995) *Mol. Cell. Biol.* **15,** 3672–3684
20. Pognonec, P., Boulokos, K. E., Gesquiere, J., Stehelin, D., and Ghysdael, J., (1988) *EMBO J.* **7,** 977–983
21. Fisher, C. L., Ghysdael, J., and Cambier, J. C. (1991) *J. Immunol.* **146,** 1743–1749
22. Jensen, K. F., Ohmstede, C.-A., Fisher, R. S., and Sahyoun, N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2850–2853
23. Enslén, H., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 20872–20877
24. Sun, P., Enslén, H., Myung, P. S., and Maurer, R. A. (1994) *Genes & Dev.* **8,** 2527–2539
25. Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) *Science* **252,** 1427–1430
26. Balazs, R. S., Kovacs, S., Cocks, W. A., Johnson, A. L., and Eayrs, J. T. (1971) *Brain Res.* **25,** 555–570
27. Schmitt, C. A., and McDonough, A. A. (1988) *J. Biol. Chem.* **263,** 17643–17649
28. Evans, R. M. (1988) *Science* **240,** 889–895
29. Almazan, G., Honegger, P., and Matthieu, J.-M. (1985) *Dev. Neurosci.* **7,** 45–54
30. Jorgensen, O. S., and Honegger, P. (1986) *Int. J. Dev. Neurosci.* **4,** 519–524
31. Corthésy-Theulaz, I., Mérillat, A.-M., Honegger, P., and Rossier, B. C. (1991) *Am. J. Physiol.* **261,** C124–C131
32. Honegger, P., Lenoir, D., and Favrod, P. (1979) *Nature* **282,** 305–307
33. Krebs, J., and Honegger, P. (1992) *Proceedings of the Eighth International Symposium on Calcium Binding Proteins and Calcium Function in Health and Disease*, Davos, Switzerland, August 23–27, 1992, (Carafoli, E., ed) p. B35
34. Krebs, J., and Honegger, P. (1995) *Proceedings of the Ninth International Symposium on Calcium Binding Proteins and Calcium Function in Health and Disease*, Arlie, VA, April 29 to May 3, 1995, (Means, A. R., and Klee, C. B., eds) p. GS 8
35. Laemmli, U. K. (1970) *Nature* **227,** 680–687
36. Zurini, M., Krebs, J., Penniston, J. T., and Carafoli, E. (1984) *J. Biol. Chem.* **259,** 618–627
37. Guerini, D., Krebs, J., and Carafoli, E. (1984) *J. Biol. Chem.* **259,** 15172–15177
38. Slaughter, G. R., Needleman, D. S., and Means, A. R. (1987) *Biol. Reprod.* **37,** 1259–1270
39. Feinberg, A., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13
40. Hanissian, H., Frangakis, M., Bland, M. M., Jawahar, S., and Chatila, T. A. (1993) *J. Biol. Chem.* **268,** 20055–20063
41. Honegger, P., and Lenoir, D. (1982) *Dev. Brain Res.* **3,** 229–238
42. Honegger, P., and Guentert, B. (1983) *Dev. Brain Res.* **11,** 245–251
43. Larson, C. A., Hsu, J.-H., Frazer, B., Chatila, T. A., and Brent, G. A. (1995) *Proceedings of the 77th Meeting of the Endocrine Society, Washington, D. C., June 14–17*, Abstr. OR1–6, p. 48, The Endocrine Society, Bethesda, MD
44. Kawaoi, A., and Tsuneda, M. (1985) *Acta Endocrinol.* **108,** 518–524