Blundell, T., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.
Brunger, A., Kuriyan, J., & Karplus, M. (1987) *Science 235*, 458–460.
Corwin, D. T., Jr., Fikar, R., & Koch, S. A. (1987) *Inorg. Chem. 26*, 3079–3080.
Corwin, D. T., Jr., Gruff, E. S., & Koch, S. A. (1988) *Inorg. Chim. Acta 151*, 5–6.
Daikun, G. P., Fairall, L., & Klug, A. (1986) *Nature 324*, 698–699.
Frankel, A. D., Bredt, D. S., & Pabo, C. O. (1988) *Science 240*, 70–73.
Garrett, T. P. J., Clingeleffer, D. J., Guss, J. M., Rogers, S. J., & Freeman, H. C. (1987) *J. Biol. Chem. 259*, 2822–2828.
Giedroc, D. P., Keating, K. M., Williams K. R., Konigsberg, W. H., & Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452–8456.
Green, L. M., & Berg, J. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4047–4051.
Ho, S. P., & DeGrado, W. F. (1987) *J. Am. Chem. Soc. 109*, 6751–6754.
Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A., & Wright, P. E. (1989) *Science 245*, 635–637.
Pabo, C. O., & Suchanek, E. G. (1986) *Biochemistry 25*, 5987–5991.
Parraga, G., Hovath, S. J., Eisen, A., Taylor, W. E., Hood, L., Young E. T., & Klevitt, R. E. (1988) *Science 241*, 1489–1492.
Regan, L., & DeGrado, W. F. (1988) *Science 241*, 976–978.
Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., & Studier, F. W. (1989) *Gene 56*, 125–135.
Schiff, L. A., Nibert, M. L., & Fields, B. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4195–4199.
Swenson, D., Baenzinger, N. C., & Coucouvanis, D. (1987) *J. Am. Chem. Soc. 100*, 1932–1934.
Vallee, B. L., & Auld, D. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 220–224.
Wells, J. A., Vasser, M., & Powers, D. B. (1985) *Gene 34*, 315–323.

Articles

Induction of Phosphoenolpyruvate Carboxykinase Gene Expression by Retinoic Acid in an Adult Rat Hepatocyte Line

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ABSTRACT: Regulation of expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene was examined in an adult rat hepatocyte line, RALA255-10G, that was immortalized with an SV40 temperature-sensitive (ts) A mutant. These hepatocytes express a transformed phenotype at the permissive temperature (33 °C) but a differentiated liver phenotype at the nonpermissive temperature (40 °C). We have shown previously that RALA255-10G cells express only low levels of liver-specific genes such as albumin and tyrosine aminotransferase at 33 °C. In the present study, we demonstrated that at 33 °C, PEPCK synthesis and mRNA expression could be detected only in the simultaneous presence of dexamethasone (DEX), retinoic acid, and dibutyryl-cAMP (Bt_2cAMP). At 40 °C, PEPCK synthesis and mRNA expression were demonstrated in the presence of Bt_2cAMP alone, but not in the presence of either DEX or retinoic acid. However, at 40 °C, PEPCK gene expression was stimulated by the combination of DEX plus retinoic acid; additionally, DEX and retinoic acid potentiated the Bt_2cAMP-mediated PEPCK induction. In RALA255-10G cells, optimal PEPCK gene expression required the simultaneous presence of DEX, retinoic acid, and Bt_2cAMP; DEX had to be present at all times. Triiodothyronine (T_3) also potentiated the Bt_2cAMP-mediated PEPCK gene expression but failed to increase further the induction by DEX/retinoic acid/Bt_2cAMP. By performing nuclear runoff assays, we demonstrated that the PEPCK gene transcription rate in the absence or presence of inducing agents was closely related to the levels of the corresponding mRNAs. At 40 °C, the PEPCK gene transcription rate in the presence of DEX/retinoic acid/Bt_2cAMP was approximately 10-fold higher than that in the presence of DEX/Bt_2cAMP, retinoic acid/Bt_2cAMP, or DEX/retinoic acid. Thus, regulation of expression of the rat PEPCK gene by retinoic acid as well as cAMP and glucocorticoid hormone is primarily regulated at the transcriptional level.

The synthesis of hepatic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK), the rate-limiting enzyme for gluconeogenesis, is under developmental and multihormonal controls [for reviews, see Hod et al. (1986) and Gluecksohn-Waelsch (1986)]. In the liver, the PEPCK gene is expressed primarily after birth, and its transcription rate is stimulated by cAMP (Lamers, et al., 1982), glucocorticoids (Magnuson et al., 1987), and thyroid hormones (Loose et al., 1985) and inhibited by insulin (Granner et al., 1983). Accordingly,

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hormonal regulation of PEPCK gene expression occurs primarily at the level of transcription. The cis-acting DNA sequences in the 5'-flanking region of the PEPCK gene that mediate cAMP and glucocorticoid induction have been defined (Wynshaw-Boris et al., 1986; Short et al., 1986; Peterson et al., 1988). Thus, the PEPCK gene provides an excellent model for studies of multihormonal regulation of tissue-specific gene expression.

Vitamin A and its derivatives are required for normal growth and differentiation (Sporn & Roberts, 1984; Chytil, 1984). In the presence of retinoic acid, an active metabolite of vitamin A, F9 teratocarcinoma stem cells differentiate into parietal endoderm (Strickland & Mahadavi, 1978), promyelocytic leukemia cells differentiate into granulocytes (Breitman et al., 1980), and developing chick limb buds establish an anterior–posterior axis (Thaller & Eichele, 1987). Retinoic acid acts through specific receptors, and these receptor genes are members of the steroid/thyroid receptor supergene family (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Krust et al., 1989; Zelent et al., 1989). Bedo et al. (1989) demonstrated that retinoic acid induces growth hormone production in pituitary cells and that it can act synergistically with thyroid and glucocorticoid hormones. However, the mechanism of action of retinoic acid is not well understood. The retinoic acid receptors have been shown to activate gene expression through the thyroid hormone responsive element (Umesono et al., 1988; Graupner et al., 1989). On the other hand, cis-acting regulatory sequences mediating retinoic acid receptor action, which are distinct from the thyroid hormone responsive element, have recently been demonstrated in the laminin B1 gene (Vasios et al., 1989) and the retinoic acid receptor β gene (de The et al., 1990). To further understand the mechanisms of retinoic acid mediated action, it is necessary to examine additional retinoic acid responsive genes. In view of the multihormonal control of liver gene expression, we examined whether retinoic acid plays a role in the expression of the rat PEPCK gene.

To study hormonal regulation of the PEPCK gene, we employed an adult rat hepatocyte line (RALA255-10G) immortalized by a simian virus 40 (SV40) A mutant virus temperature-sensitive (ts) in the gene required for maintenance of transformation (Chou, 1983). At the permissive temperature (33 °C), RALA255-10G cells produce a functional T antigen, express a transformed phenotype, and synthesize low levels of liver marker proteins. When transferred to the nonpermissive temperature (40 °C), the T antigen becomes inactive, and the cells regain the normal, differentiated phenotype. This is manifested by the synthesis of greatly increased levels of liver marker proteins such as albumin, transferrin, and tyrosine aminotransferase (Chou, 1983; Chou & Yeoh, 1987).

In the present study, we examined expression of the PEPCK gene in RALA255-10G hepatocytes. As expected, PEPCK synthesis is primarily observed in cells grown at the nonpermissive temperature and in the presence of glucocorticoid hormone and cAMP. In addition, we demonstrated that retinoic acid greatly enhances the glucocorticoid/cAMP-mediated induction of PEPCK gene expression.

Materials and Methods

Cell Culture. The RALA255-10G cell line (Chou, 1983) was grown and maintained in α-modified minimal essential medium supplemented with dexamethasone (DEX, 0.1 μM), streptomycin (100 μg/mL), penicillin (100 units/mL), and 4% fetal bovine serum. Cells were grown initially at 33 °C (permissive temperature) for 3–5 days until reaching confluence, and then some of the cultures were shifted to 40 °C (nonpermissive temperature) for further experimentation. Medium was changed every 2 days.

Nucleic Acid Hybridization Analysis. Total RNA was extracted by the guanidinium thiocyanate/CsCl method (Chirgwin et al., 1979), and poly(A+) RNA was purified by oligo(dT)–cellulose chromatography. RNA was separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach et al., 1977) and transferred to Zetabind membranes (AMF, Meriden, CT) by electroblotting. The antisense riboprobes were synthesized from pGEM (Promega Biotech, Madison, WI) subclones of cDNAs encoding rat β-actin (B. Patterson, National Institutes of Health, Bethesda, MD), rat PEPCK (Yoo-Warren et al., 1983), rat glucocorticoid receptor (Miesfled et al., 1986), or human α- (Giguere et al., 1987) and β- (Benbrook et al., 1988) retinoic acid receptors. Hybridization in the presence of dextran sulfate and washing were performed as described (Chou & Yeoh, 1987). RNA blots were washed twice in 2X SSC (SSC is 150 mM sodium chloride/15 mM sodium citrate) containing 0.5% SDS for 30 min each at room temperature, and then 4 times in 0.1X SSC containing 0.1% SDS for 60 min each at 65 °C.

Biosynthesis of PEPCK. Cultures were labeled for 3 h at either 33 °C or 40 °C by incubation in methionine-free medium to which L-[^{35}S]methionine at 100 μCi/mL (ICN Biochemicals, Inc., Lisle, IL) was added. Anti-PEPCK-precipitable polypeptides in cell lysates were isolated by immunoprecipitation with goat antiserum to rat PEPCK in the presence of carrier PEPCK and analyzed by polyacrylamide–SDS gel electrophoresis (Laemmli, 1970) and fluorography. Apparent molecular weights were determined by using [^{14}C]-methionine-labeled protein standards obtained from Amersham Corp. (Arlington Heights, IL).

Transcription Assay. Nuclei were isolated essentially as described by Clayton and Darnell (1983). Briefly, cells were homogenized at 4 °C in 10 volumes of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3.5 mM $MgCl_2$, 150 mM KCl, 14 mM 2-mercaptoethanol, and 0.5% Nonident P40. Nuclei were pelleted by centrifugation at 1000g for 5 min, stored [(2–4) $\times 10^7$ nuclei/mL] in 20 mM Tris-HCl, pH 7.4, 4 mM $MnCl_2$, 1 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM spermidine, and 60% glycerol, and used within 1 week after isolation. Rates of RNA transcription were measured with 250 μCi of [^{32}P]-UTP (3000 Ci/mmol; New England Nuclear Products, Boston MA) in 40 mM Tris-HCl, pH 8.3, 150 mM NH_4Cl, 7.5 mM $MgCl_2$, and 1 mM each of ATP, CTP, and GTP containing (2–4) $\times 10^7$ nuclei in a total volume of 400 μL and incubated at 27 °C for 35 min. α-Amanitin, when present, was at 1 μg/mL. RNA synthesis was terminated by incubating with RNase-free DNase (62.5 μg/mL) for 15 min at 37 °C followed by incubation with proteinase K (1 mg/mL), heparin (3 mg/mL), 10 mM Tris-HCl, pH 7.4, 15 mM EDTA, and 3% SDS for 3 h at 42 °C. RNA was purified by phenol/chloroform extraction, trichloroacetic acid and alcohol precipitation, and DNase treatment and then solubilized in 10 mM Tris-HCl, pH 8, and 1 mM EDTA and used for hybridization to solid-phase plasmid DNA.

Plasmid pUC19 (10 μg/slot) or an equal amount of PEPCK genomic subclones RPEPCKG-BH5 (containing exons 1 and 2) and RPEPCKG-HS3 (containing exons 3–8 and part of exon 9) (Yoo-Warren et al., 1983) were boiled in 0.2 N NaOH for 5 min, adjusted to 6× SSC, and applied onto a Zetabind membrane using a slot-blot (Manifold II, Schleicher & Schuell, Keene, NH). Filters were UV-cross-linked with a Stratagene cross-linker (Stratagene Cloning Systems, La Jolla,

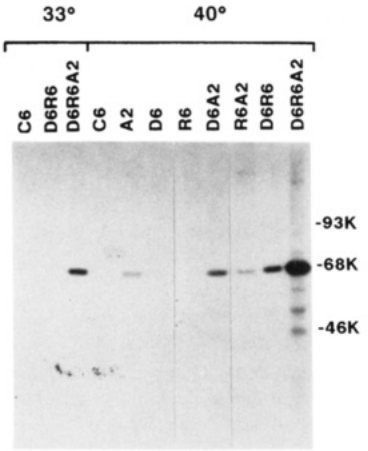

FIGURE 1: Effects of DEX, retinoic acid, and Bt_2cAMP on PEPCK biosynthesis. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were washed 3 times with PBS and treated with control medium (C) or medium containing 0.1 μM DEX (D), 10 μM retinoic acid (R), 0.5 mM Bt_2cAMP (A), DEX/Bt_2cAMP (DA), retinoic acid/Bt_2cAMP (RA), DEX/retinoic acid (DR), or DEX/retinoic acid/Bt_2cAMP (DRA). Some of the cultures were kept at 33 °C while others were shifted to 40 °C for an additional 6-days growth. DEX or retinoic acid was added on day 0 (day 0–6); Bt_2cAMP was added the last 2 days (day 4–6). The PEPCK proteins in cell lysates were isolated by immunoprecipitation and analyzed by SDS–polyacrylamide gel electrophoresis as described under Materials and Methods.

CA) and hybridized for 72 h at 42 °C in 5× SSC, 50% formamide, 10% dextran sulfate, 1% SDS, 0.02% each of Ficoll, poly(vinylpyrrolidone), and bovine serum albumin, 200 μg/mL sonicated salmon sperm DNA, 100 μg/mL poly(A), and 5×10^6 cpm/mL labeled nuclear RNA. After hybridization, filters were washed as described for Northern filters.

Results

Induction of PEPCK Synthesis by Bt_2cAMP, DEX, and Retinoic Acid. We have previously shown that RALA255-10G adult rat hepatocytes are temperature-sensitive for maintenance of transformation (Chou, 1983; Chou & Yeoh, 1987). At the permissive temperature (33 °C), these cells express a transformed phenotype and synthesize low levels of liver-specific proteins. However, at the nonpermissive temperature (40 °C), RALA255-10G cells express a differentiated liver phenotype, and synthesis of liver-specific proteins such as albumin, transferrin, and tyrosine aminotransferase is greatly induced. In the present study, we examined biosynthesis of PEPCK, a key enzyme for gluconeogenesis, in RALA255-10G cells grown at the permissive and nonpermissive temperatures. At 33 °C, PEPCK synthesis was not observed in control cultures or in cultures grown in the presence of either DEX, retinoic acid, Bt_2cAMP, or a combination of any two of the agents. Data for control and DEX plus retinoic acid are shown in Figure 1. However, in the simultaneous presence of DEX/retinoic acid/Bt_2cAMP at 33 °C, RALA255-10G synthesized significant amounts of the PEPCK polypeptide (Figure 1). Like albumin (Chou, 1983) and tyrosine aminotransferase (Chou & Yeoh, 1987), the synthesis of PEPCK was greatly induced after shifting these adult rat hepatocytes to 40 °C (Figure 1). At 40 °C, PEPCK synthesis was undetectable in the presence of DEX or retinoic acid alone, but was observed in the presence of either Bt_2cAMP, DEX/Bt_2cAMP, retinoic acid/Bt_2cAMP, or DEX/retinoic acid. The simultaneous addition of all three agents at 40 °C gave a synergistic induction (Figure 1).

In agreement with PEPCK biosynthesis results, expression

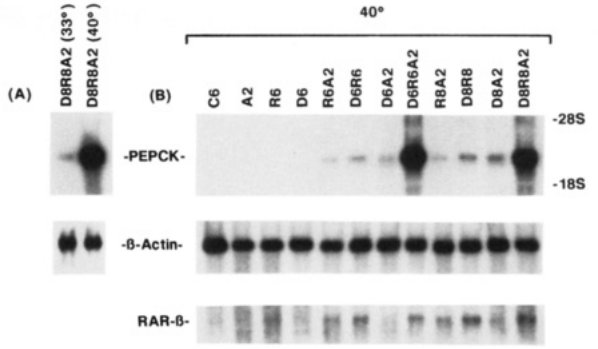

FIGURE 2: Effects of DEX, retinoic acid, and Bt_2cAMP on PEPCK mRNA expression. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were washed 3 times with PBS and treated with control medium (C) or medium containing 0.1 μM DEX (D), 10 μM retinoic acid (R), 0.5 mM Bt_2cAMP (A), DEX/Bt_2cAMP (DA), retinoic acid/Bt_2cAMP (RA), DEX/retinoic acid (DR), or DEX/retinoic acid/Bt_2cAMP (DRA). Some of the cultures were kept at 33 °C while others were shifted to 40 °C. (A) RNAs were isolated from cultures which had been grown for an additional 8 days in the presence of DEX/retinoic acid/Bt_2cAMP at 33 °C or 40 °C. (B) RNAs were isolated from cultures which had been grown for an additional 6 or 8 days at 40 °C in the presence of the various hormones as indicated. DEX or retinoic acid was added on day 0 (day 0–6 for 6-day cultures or day 0–8 for 8-day cultures); Bt_2cAMP was added the last 2 days (day 4–6 for 6-day cultures or day 6–8 for 8-day cultures). Poly(A+) RNAs (5 μg/lane) were separated by formaldehyde–agarose gel electrophoresis and transferred to Zetabind membranes. Filters were hybridized to an antisense PEPCK or RAR-β riboprobe as described under Materials and Methods. After removal of bound PEPCK by washing the filters in 95% formamide (60 °C for 30 min), the filters were rehybridized with a β-actin riboprobe.

of the PEPCK gene at 33 °C could be detected only in the presence of DEX/retinoic acid/Bt_2cAMP (Figure 2A). PEPCK mRNA levels in cultures grown in the presence of DEX/retinoic acid/Bt_2cAMP at 33 °C were approximately 5% of the levels of the corresponding cultures grown at 40°C.

PEPCK gene expression was also greatly stimulated in RALA255-10G cells at 40 °C (Figure 2B). After 6-days incubation at 40 °C, expression of PEPCK mRNA was observed in the presence of retinoic acid/Bt_2cAMP, DEX/retinoic acid, and DEX/Bt_2cAMP; expression was further increased after 2 additional days at 40 °C (day 8, Figure 2B). After a longer exposure, PEPCK mRNA could also be detected in the presence of Bt_2cAMP alone (data not shown). The simultaneous addition of DEX/retinoic acid/Bt_2cAMP gave a synergistic stimulation of approximately 10-fold above the levels observed in the presence of retinoic acid/Bt_2cAMP, DEX/retinoic acid, or DEX/Bt_2cAMP on both day 6 and day 8 (Figure 2B). Maximal PEPCK gene expression in the presence all three agents could be maintained for at least 8 days at the nonpermissive temperature (Figure 2B).

Three types of retinoic acid receptor genes (α, β, and γ) have been isolated from human (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Krust et al., 1989) and mouse (Zelent et al., 1989). Although the DNA sequences of the three retinoic acid receptors differ from each other, sequences within each type are well conserved among species. de The et al. (1989) reported that in PCL/PRF/5 human hepatoma cells, the β-retinoic acid receptor gene was transcriptionally up-regulated by retinoic acid. Moreover, in F9 tetratocarcinoma cells, only the β-retinoic acid receptor mRNA expression was increased after retinoic acid addition (Hu & Gudas, 1990). In view of the sequence conservation among the retinoic acid mRNA between species, we examined expression of the retinoic acid receptor mRNA in

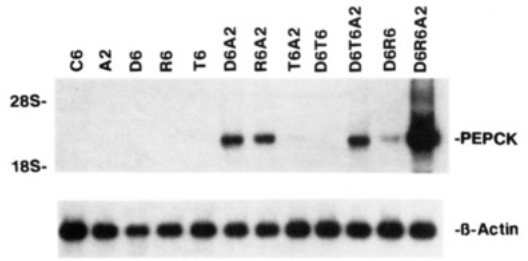

FIGURE 3: Effects of thyroid hormone on PEPCK mRNA expression. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were washed 3 times with PBS, were treated with control medium (C) or medium containing 0.1 μM DEX (D), 10 μM retinoic acid (R), 0.5 mM Bt_2cAMP (A), 0.01 μM T_3 (L-3,3′,5-triiodothyronine, T), DEX/Bt_2cAMP (DA), retinoic acid/Bt_2cAMP (RA), T_3/Bt_2cAMP (TA), DEX/retinoic acid (DR), DEX/T_3 (DT), DEX/T_3/Bt_2cAMP (DTA), or DEX/retinoic acid/Bt_2cAMP (DRA), and were shifted to 40 °C for an additional 6 days of growth. DEX, retinoic acid, or T_3 was added on day 0 (day 0–6); Bt_2cAMP was added the last 2 days (day 4–6). Poly(A^+) RNAs (5 μg/lane) were separated by formaldehyde–agarose gel electrophoresis and transferred to Zetabind membranes. Filters were hybridized to an antisense PEPCK riboprobe as described under Materials and Methods. After removal of bound PEPCK by washing the filters in 95% formamide (60 °C for 30 min), the filters were rehybridized with a β-actin riboprobe.

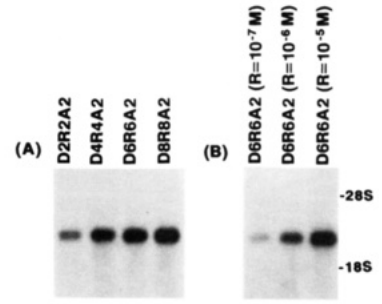

FIGURE 4: (A) Time course of PEPCK mRNA expression at 40 °C in the presence of DEX/retinoic acid/Bt_2cAMP. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were changed to medium containing DEX (D, 0.1 μM), retinoic acid (R, 10 μM), and Bt_2cAMP (A, 0.5 mM) and were shifted to 40 °C (day 0). Cells were harvested for RNA isolation after an additional 2-, 4-, 6-, or 8-days growth at 40 °C. DEX or retinoic acid was present at all times; Bt_2cAMP was present only for the last 2 days. (B) Optimal concentration of retinoic acid for PEPCK mRNA expression. The culture conditions were the same as (A) except that the retinoic acid concentrations were varied. Cultures were incubated at 40 °C for 6 days. Poly(A^+) RNAs (5 μg/lane) were separated by formaldehyde–agarose gel electrophoresis and transferred to Zetabind membranes. Filters were hybridized to an antisense PEPCK riboprobe as described under Materials and Methods.

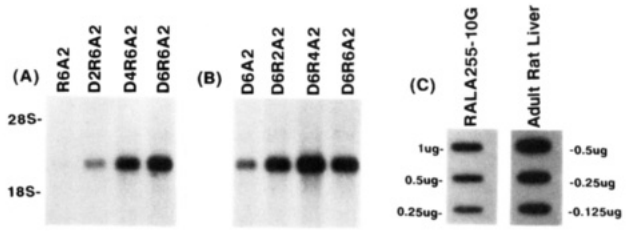

FIGURE 5: Conditions for optimal PEPCK mRNA expression in the presence of DEX, retinoic acid, and Bt_2cAMP. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were shifted to 40 °C. (A) Effects of DEX. Cultures were treated with medium containing DEX (D, 0.1 μM), retinoic acid (R, 10 μM), and Bt_2cAMP (A, 0.5 mM) and were incubated for an additional 6 days at 40 °C. Bt_2cAMP was present for 2 days (day 4–6), and DEX was present for 2 (day 4–6, D2R6A2), 4 (day 2–6, D4R6A2), or 6 (Day 0–6, D6R6A2) days. (B) Effects of retinoic acid. Bt_2cAMP was present for 2 days (day 4–6), and retinoic acid was present for 2 (day 4–6, D6R2A2), 4 (day 2–6, D6R4A2), or 6 (day 0–6, D6R6A2) days. Poly(A^+) RNAs (5 μg/lane) were separated by formaldehyde–agarose gel electrophoresis and transferred to Zetabind membranes. Filters were hybridized to an antisense PEPCK riboprobe as described under Materials and Methods. (C) Quantification of PEPCK mRNA in RALA255-10G cells grown at 40 °C for 6 days in the presence of DEX/retinoic acid/Bt_2cAMP. DEX or retinoic acid was added on day 0, but Bt_2cAMP was added on day 4. Poly(A^+) RNA from adult rat liver or RALA255-10G cells was applied onto a Zetabind membrane using a slot-blot (Schleicher & Schuell, Manifold II) and hybridized to an antisense PEPCK riboprobe as described under Materials and Methods. The RNAs were quantified by scanning the autoradiograms of different exposures with the Bio-Rad densitometer (Bio-Rad, Richmond, CA).

RALA255-10G hepatocytes using probes derived from the human receptors. Our results show that the β- (Figure 2B) but not the α- (data not shown) retinoic acid receptor mRNA was stimulated by retinoic acid.

It has previously been demonstrated that expression of the PEPCK gene can be induced by thyroid hormone (Loose et al., 1985). Moreover, the retinoic acid receptor can bind to the thyroid hormone responsive element and mediate gene activation (Umesono et al., 1988). We examined the role of thyroid hormone in modulating PEPCK gene expression in RALA255-10G cells (Figure 3). Thyroid hormone by itself or in combination with DEX could not stimulate PEPCK gene expression, but did potentiate the Bt_2cAMP-mediated PEPCK induction. However, thyroid hormone was a much weaker inducer of the PEPCK gene expression than retinoic acid (Figure 3). In addition, thyroid hormone did not further enhance induction of the PEPCK gene expression by retinoic acid, either in the absence or in the presence of DEX and Bt_2cAMP (data not shown).

Characterization of the DEX/Retinoic Acid/Bt_2cAMP-Mediated Induction. To analyze the DEX/retinoic acid/Bt_2cAMP-mediated induction of the PEPCK gene, PEPCK mRNA levels in RALA255-10G cells were examined over time. Maximal expression in the presence of all three agents was observed after a 6–8-day incubation at 40 °C (Figure 4A). Earlier studies (Chou, 1983; Chou & Yeoh, 1987) have characterized the concentrations of DEX and Bt_2cAMP required for maximal induction of liver gene expression in RALA255-10G cells. Now, we demonstrate that the optimal concentration of retinoic acid for stimulating PEPCK mRNA expression was 10^{-5} M (Figure 4B).

Although DEX or retinoic acid alone could not induce PEPCK gene expression at 40 °C, the continued presence of DEX was necessary for maximal PEPCK mRNA expression in the presence of DEX/retinoic acid/Bt_2cAMP (Figure 5A). Conversely, the presence of Bt_2cAMP and retinoic acid was not required at all times. To achieve optimal PEPCK mRNA expression in RALA255-10G cells in the presence of DEX/retinoic acid/Bt_2cAMP at 40 °C, Bt_2cAMP was only required for the last 2 days (day 4 to day 6 for a 6-day incubation). Exposure of cells to Bt_2cAMP for 4 days (day 2 to day 6) failed to further increase PEPCK mRNA levels (data not shown). With regard to retinoic acid, optimal PEPCK gene expression was observed in cultures grown in the presence of DEX/retinoic acid/Bt_2cAMP at 40 °C when retinoic acid was present for 4 days (day 2 to day 6) (Figure 5B).

RALA255-10G cells exhibit a differentiated liver phenotype at 40 °C. However, the PEPCK mRNA level in cells grown at 40 °C for 6 days under these optimal conditions (in the presence of DEX/retinoic acid/Bt_2cAMP) was about 6% of the adult rat liver level (Figure 5C).

Expression of the PEPCK Gene Is Mainly Regulated at the Transcriptional Level. The observed changes in the levels

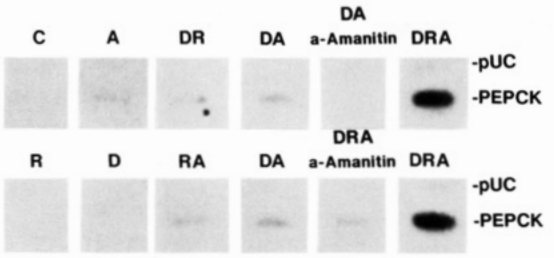

FIGURE 6: Effects of DEX, retinoic acid, and Bt_2cAMP on the transcriptional activity of the PEPCK gene. RALA255-10G cells were initially grown at 33 °C in medium containing 0.1 μM DEX. After 4–5-days growth at 33 °C (reaching confluence, day 0), cultures were washed 3 times with PBS, were treated with control medium (C) or medium containing 0.1 μM DEX (D), 10 μM retinoic acid (R), 0.5 mM Bt_2cAMP (A), DEX/Bt_2cAMP (DA), retinoic acid/Bt_2cAMP (RA), DEX/retinoic acid (DR), or DEX/retinoic acid/Bt_2cAMP (DRA), and were shifted to 40 °C. Nuclei were isolated from cultures which had been grown at 40 °C for 6 days. Rates of RNA transcription were determined as described under Materials and Methods. α-Amanitin, when present, was at 1 μg/mL. Data for DA and DRA are shown in duplicate for comparison.

of PEPCK mRNAs may be the consequence of an increased rate of gene transcription or a change in mRNA stability. To distinguish between these alternatives, the transcription rates of the PEPCK gene were determined in isolated nuclei prepared from RALA255-10G cells grown under various experimental conditions. Low rates of PEPCK gene transcription were detected in the presence of Bt_2cAMP, DEX/Bt_2cAMP, retinoic acid/Bt_2cAMP, and DEX/retinoic acid (Figure 6). However, in the simultaneous presence of DEX/retinoic acid/Bt_2cAMP, the PEPCK gene transcription rate was increased approximately 10-fold (Figure 6). This increase in PEPCK transcription rate paralleled the corresponding increase in the steady-state level of PEPCK mRNA. PEPCK gene transcription was sensitive to α-amanitin, which markedly repressed the PEPCK gene transcription rate (Figure 6).

Discussion

In the present study, we demonstrate that the optimal expression of the gluconeogenic enzyme, PEPCK, in the RALA255-10G adult rat hepatocyte line requires the simultaneous presence of glucocorticoid hormone, retinoic acid, and Bt_2cAMP. These agents stimulate the PEPCK gene transcription rate, resulting in increased mRNA expression and enzyme synthesis. Previous studies have shown that control of PEPCK gene expression by glucocorticoids or cAMP occurs mainly at the level of gene transcription. We now show that retinoic acid also plays a role in modulating PEPCK synthesis; this retinoid greatly potentiates the induction of PEPCK gene transcription by glucocorticoid hormone and cAMP.

In the rat PEPCK gene, two glucocorticoid responsive elements (GREs) and one cAMP regulatory element (CRE) are located in the 5′-flanking regions of this gene, and transcriptional activation of the PEPCK gene by glucocorticoid and cAMP has been confirmed by gene transfer experiments (Short et al., 1986; Peterson et al., 1988). The demonstration that PEPCK gene expression is also transcriptionally regulated by retinoic acid suggests that a functional retinoic acid responsive element(s) (RARE) that modulates retinoic acid action may be present in the rat PEPCK gene. The presence of a RARE in the 5′-flanking region of the laminin B1 gene (Vasios et al., 1989) and the retinoic acid receptor β gene (de The et al., 1990) has recently been demonstrated. Both studies indicate that the three types of retinoic acid receptors recognize the same RARE, mediating trans-activation of gene transcription. It has also been shown that the retinoic acid receptor may modulate gene expression by binding to the thyroid hormone responsive element of target genes (Umesono et al., 1988; Graupner et al., 1989). However, the sequences of the two identified RAREs differ from the consensus sequence of the thyroid hormone responsive element. Moreover, the laminin B1 gene could not be activated by thyroid hormone (Vasios et al., 1989). In the present study, thyroid hormone could only potentiate the Bt_2cAMP-mediated activation of PEPCK gene expression in RALA255-10G hepatocytes. Moreover, thyroid hormone was at least 10 times less potent in stimulation of PEPCK mRNA expression than retinoic acid. Our results suggest that the RARE of the rat PEPCK gene, like the laminin B1 gene, is probably functionally distinct from the thyroid hormone responsive element.

As mentioned above, maximal induction of PEPCK gene expression was observed in RALA255-10G cells in the simultaneous presence of DEX, retinoic acid, and Bt_2cAMP. However, glucocorticoid hormone was the only agent which had to be present at all times for maximal expression of this gene. This steroid has been shown to induce expression of a variety of liver-specific genes including albumin (Chou, 1983; Nawa et al., 1986), tyrosine aminotransferase (Nickol et al., 1978; Chou & Yeoh, 1987), transferrin (Chou, 1983), and α_1-acid glycoprotein (Reinke & Feigelson, 1985). Moreover, RALA255-10G cells grown in medium lacking glucocorticoids decrease or lose the ability to express many liver-specific genes (Chou, 1983; Chou & Yeoh, 1987). Taken together, these studies indicate that optimal differentiation of hepatocytes requires the glucocorticoid hormone.

It has been established that expression of differentiated liver genes in SV40 tsA mutant-transformed hepatocytes can be induced by shifting these cells to the nonpermissive temperature of 40 °C [for a review, see Chou (1989)]. In the RALA255-10G adult hepatocytes, albumin and tyrosine aminotransferase gene expression is markedly stimulated at 40 °C (Chou, 1983; Chou & Yeoh, 1987). As expected, expression of the PEPCK gene in these cells is also temperature-sensitive. RALA255-10G cells express low levels of PEPCK gene at the permissive temperature of 33 °C. Shifting these cells to 40 °C greatly induced PEPCK synthesis and mRNA expression. This induction is regulated primarily at the level of gene transcription. The coordinate induction of most, if not all, liver-specific genes at the nonpermissive temperature implies that one or more trans-acting regulators are induced or repressed during the temperature shift.

The molecular mechanisms responsible for the potentiation of cAMP-mediated and glucocorticoid hormone mediated PEPCK gene induction by retinoic acid are not known. It is possible that binding of the retinoic acid–receptor complex to the control regions of the target gene alters the binding of the glucocorticoid–receptor complex or the CRE/AP-2-like binding protein to their respective binding regions, causing either positive or negative regulation. Several lines of evidence support this mode of retinoic acid action. First, retinoic acid potentiates glucocorticoid and thyroid hormone induction of the growth hormone gene (Bedo et al., 1989). Second, binding of the glucocorticoid receptor to nonadjacent sites of the rat tyrosine aminotransferase gene synergistically enhances its expression (Jantzen et al., 1987). Third, glucocorticoids negatively regulate human chorionic gonadotropin α-gene expression by interfering with other transcription factors (Akerblom et al., 1988). Whether retinoic acid potentiates the PEPCK gene by increasing binding of the hormone–receptor complex or CRE/AP-2-like binding protein to its

discrete nuclear sequences remains to be determined.

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Registry No. PEPCK, 9013-08-5; DEX, 50-02-2; Bt_2cAMP, 362-74-3; T_3, 6893-02-3; retinoic acid, 302-79-4.

REFERENCES

Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D., & Mellon, P. L. (1988) *Science 241*, 350–353.

Bedo, G., Santisteban, P., & Aranda, A. (1989) *Nature 339*, 231–234.

Benbrook, D., Lernhardt, E., & Pfahl, M. (1988) *Nature 333*, 669–672.

Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P., & Dejean, A. (1988) *Nature 332*, 850–853.

Breitman, T., Selonick, S., & Collins, S. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2936–2940.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chou, J. Y. (1983) *Mol. Cell. Biol. 3*, 1013–1020.

Chou, J. Y. (1989) *Mol. Endocrinol. 3*, 1511–1514.

Chou, J. Y., & Yeoh, G. C. T. (1987) *Cancer Res. 47*, 5415–5420.

Chytil, F. (1984) *Pharmacol. Rev. 36 (Suppl. 2)*, 935–1005.

Clayton, D. F., & Darnell, J. E., Jr. (1983) *Mol. Cell. Biol. 3*, 1552–1561.

de The, H., Marchio, A., Tiollais, P., & Dejean, A. (1989) *EMBO J. 8*, 429–433.

de The, H., de Mar Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H., & Dejean, A. (1990) *Nature 343*, 177–180.

Giguere, V., Ong, E. S., Segui, P., & Evans, R. M. (1987) *Nature 330*, 624–629.

Gluecksohn-Waelsch, S. (1986) *Ann. N.Y. Acad. Sci. 478*, 101–108.

Granner, D., Andreone, T., Sasaki, K., & Beale, E. (1983) *Nature 305*, 549–551.

Graupner, G., Wills, K. N., Tzukerman, M., Zhang, X.-K., & Pfahl, M. (1989) *Nature 340*, 653–656.

Hod, Y., Cook, J. S., Weldon, S. L., Short, J. M., Wynshaw-Boris, A., & Hanson, R. W. (1986) *Ann. N.Y. Acad. Sci. 478*, 31–45.

Hu, L., & Gudas, L. J. (1990) *Mol. Cell. Biol. 10*, 391–396.

Jantzen, H.-M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., & Schutz, G. (1987) *Cell 49*, 29–38.

Krust, A., Kastner, P., Petkovich, M., Zelent, A., & Chambon, P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5310–5314.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lamers, W. H., Hanson, R. W., & Meisner, H. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5137–5141.

Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.

Loose, D. S., Cameron, D. K., Short, H. P., & Hanson, R. W. (1985) *Biochemistry 24*, 4509–4512.

Magnuson, M. A., Quinn, P. G., & Granner, D. K. (1987) *J. Biol. Chem. 262*, 14917–14920.

Miesfled, R., Rusconi, S., Godowski, P. M., Maler, B. A., Okret, S., Wikstrom, A.-C., Gustafsson, J.-A., & Yamamoto, K. R. (1986) *Cell 46*, 389–399.

Nawa, K., Nakamura, T., Kumatori, A., Noda, C., & Ichihara, A. (1986) *J. Biol. Chem. 261*, 16883–16888.

Nickol, J. M., Lee, K.-L., & Kenney, F. T. (1978) *J. Biol. Chem. 253*, 4009–4015.

Peterson, D. D., Magnuson, M. A., & Granner, D. K. (1988) *Mol. Cell. Biol. 8*, 96–104.

Petkovich, M., Brand, N. J., Krust, A., & Chambon, P., (1987) *Nature 330*, 444–450.

Reinke, R., & Feigelson, P. (1985) *J. Biol. Chem. 260*, 4397–4403.

Short, J. M., Wynshaw-Boris, A., Short, H. P., & Hanson, R. W. (1986) *J. Biol. Chem. 261*, 9721-9726.

Sporn, M. B., & Roberts, A. B. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) Vol. 1, pp 235–279, Academic Press, New York.

Strickland, S., & Mahadavi, V. (1978) *Cell 15*, 393–403.

Thaller, C., & Eichele, G. (1987) *Nature 327*, 625–628.

Umesono, K., Giguere, V., Glass, C. K., Rosenfeld, M. G., & Evans, R. M. (1988) *Nature 336*, 262–265.

Vasios, G. W., Gold, J. D., Petkovich, M., Chambon, P., & Gudas, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9099–9103.

Wynshaw-Boris, A., Short, J. M., Loose, D. S., & Hanson, R. W. (1986) *J. Biol. Chem. 261*, 9714–9720.

Yoo-Warren, H., Monahan, J. E., Short, J., Short, H., Bruzel, A., Wynshaw-Boris, A., Meisner, H. M., Samols, D., & Hanson, R. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3656–3660.

Zelent, A., Krust, A., Petkovich, M., Kastner, P., & Chambon, P. (1989) *Nature 339*, 714–717.