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Interactions of Thyroid Hormone, Growth Hormone, and High Carbohydrate, Fat-Free Diet in Regulating Several Rat Liver Messenger Ribonucleic Acid Species†

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ABSTRACT: Pleiotropic effects of thyroid hormone, growth hormone, and high carbohydrate, fat-free diet in regulating the intracellular levels of rat liver mRNA were examined. Total hepatic poly(A)-containing RNA, isolated from appropriately treated animals, was translated in the mRNA-dependent reticulocyte lysate system. Two-dimensional gel electrophoresis of the ^{35}S-labeled translational products allowed separation of approximately 200 different mRNA-encoded products. Computerized video densitometry was utilized to quantitate the relative proportion of any individual product in the total population. Previous work [Seelig, S., Liaw, C., Towle, H. C., & Oppenheimer, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4733–4737] has shown that administration of triiodothyronine (T_3) to hypothyroid rats leads to specific alterations in approximately 8% of the mRNA species detected by this technique. We now show that a subset of these T_3-responsive mRNA species is indirectly affected by T_3 through its action on pituitary production of growth hormone. Administration of growth hormone to hypothyroid rats leads to specific alterations in the levels of eight different mRNA species. The relative levels of four mRNA species are diminished, and those of four mRNA species are increased by growth hormone. Thus, this hormone which presumably binds to a membrane receptor can act at a pretranslational level to augment and attenuate several specific mRNA sequences. For two of these mRNA species, the combined action of T_3 and growth hormone is necessary for maintenance of normal levels. In addition, switching rats from standard laboratory chow to a diet high in carbohydrate and fat free resulted in changes in the levels of 10 different mRNA-encoded products. Interestingly, all but one of these mRNA species was also influenced by the thyroidal status of the animal. Thus, a high degree of overlap exists between mRNA species regulated by T_3 and a high carbohydrate, fat-free diet. Most T_3-responsive mRNA species appear to be regulated in a complex multifactorial pathway.

Many, if not all, of the cellular actions of thyroid hormone are initiated by binding of triiodothyronine (T_3)[1] to a specific chromatin-bound receptor in target cells [for reviews, see Samuels (1978), Latham et al. (1978), and Oppenheimer (1979)]. This hormone–receptor interaction is postulated to cause specific alterations in the nuclear production of RNA (Tata & Widnell, 1966) and consequently in the pattern of proteins synthesized in the tissue. In no case has the expression of a gene product been definitively demonstrated to be directly responsive to hormone–receptor binding; however, the finding of increased mRNA levels for at least three specific proteins induced by T_3 provides correlative evidence in favor of this pathway. Thus, the inductions of growth hormone in rat pituitary tumor cell lines (Martial et al., 1977a; Seo et al., 1977; Shapiro et al., 1978), α_{2u}-globulin in rat liver (Roy et al., 1976; Kurtz et al., 1976), and malic enzyme in avian and rat liver (Towle et al., 1981; Siddiqui et al., 1981) are all accompanied by parallel changes in the cellular levels of mRNA encoding these proteins.

Recently we have examined more fully the qualitative and quantitative range of the effects of T_3 on the mRNA popu-

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[1] Abbreviations: T_3, L-triiodothyronine; CHO diet, high carbohydrate, fat-free diet; Gdn·HCl, guanidine hydrochloride.

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lation of rat liver (Seelig et al., 1981). Total hepatic poly(A)-containing RNA was isolated from rats of hypothyroid, euthyroid, and hyperthyroid states. These mRNA populations were analyzed by in vitro translation in the mRNA-dependent reticulocyte lysate system in the presence of [^{35}S]methionine, followed by two-dimensional gel electrophoresis (O'Farrell, 1975) of the ^{35}S-labeled translational products. In this manner, approximately 230 translational products corresponding to specific mRNA sequences could be individually monitored. Changes in the thyroid status of the rat resulted in altered mRNA levels for 20 specific products. Thus, approximately 8% of the mRNA species analyzed were in the "T_3 domain" of liver.

Several of the thyroid hormone-induced responses of rat liver have been shown to be influenced by other hormonal and metabolic factors. For example, hepatic malic enzyme is induced by changing the diet of the animal from standard laboratory chow to a high carbohydrate, fat-free (CHO) diet (Tepperman & Tepperman, 1964; Wise and Ball, 1964). As observed for the T_3 induction of this enzyme, levels of mRNA coding for malic enzyme are increased in parallel with enzyme activity during the dietary induction (Towle et al., 1980). The rat hepatic protein α_{2u}-globulin requires the simultaneous presence of testosterone, glucocorticoid, and growth hormone as well as thyroid hormone for its efficient production (Roy, 1973; Kurtz & Feigelson, 1978). In the absence of any one of these four hormones, levels of mRNA encoding α_{2u}-globulin are reduced to undetectable levels.

In this paper, we have taken advantage of the technique of two-dimensional gel electrophoresis of mRNA-encoded translational products to more fully characterize the interactions of thyroid hormone and other factors in regulating hepatic mRNA levels. Of particular interest was the effect of growth hormone on the hepatic mRNA population, since its production in the pituitary is known to be influenced by plasma T_3 levels (Solomon & Greep, 1959; Hervas et al., 1975). We demonstrate that 8 of the 20 T_3-responsive mRNA sequences previously described are responsive to the administration of growth hormone to hypothyroid animals. In addition, the feeding of CHO diet to rats was found to alter the levels of 10 mRNA sequences, and, in all cases but one, these same mRNA species were responsive to T_3. Thus, the interaction of T_3 with other hormonal and metabolic factors appears to be a generalized phenomenon of its regulation of rat liver mRNA.

Experimental Procedures

Male Sprague-Dawley rats weighing approximately 250 g were used. Animals were rendered hypothyroid by surgical thyroidectomy and ^{131}I ablation. Ovine growth hormone (NIH-GH-S11; 0.56 IU/mg) was the gift of the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases and was injected subcutaneously at a dose of 0.2 IU per 100 g body weight per day in saline solution, pH 8.0. This growth hormone preparation contains the following levels of contaminating pituitary hormones: prolactin, 0.5 unit/mg; TSH, <0.05 unit/mg; LH, <0.025 unit/mg; FSH, <0.02 unit/mg; ACTH, not detectable. T_3 was administered intraperitoneally at the doses indicated in individual experiments. CHO diet was the "Fat Free Test Diet" from ICN Pharmaceuticals which contains 58% sucrose (by weight) and no fats.

Extraction of poly(A)-containing RNA, translation in the mRNA-dependent rabbit reticulocyte lysate, and two-dimensional electrophoresis of ^{35}S-labeled translational products were carried out as described (Seelig et al., 1981) with minor modifications. The isoelectric gradient contained 1.2% pH 5–8 and 0.8% pH 3.5–10 Ampholines (LKB). In all experiments, an equivalent number of total trichloroacetic acid precipitable cpm (usually 200 000) was loaded onto each gel. Isoelectric-focused gels were equilibrated for 30 min rather than 1 h before electrophoresis in the second dimension. RNA from rat kidneys was extracted by using minor modifications of the Gdn·HCl method (Deeley et al., 1977; Chirgwin et al., 1979).

Quantitation of individual spots on radiofluorograms of the two-dimensional gels was carried out by using a system developed in this laboratory (Mariash et al., 1982). This system includes a medium resolution black and white video camera for data acquisition, a video digitizing circuit for data translation, and a microcomputer for data analysis. The ability of this system to accurately determine cpm for individual spots was determined by comparing results to cpm obtained by cutting and directly counting corresponding areas. The regression between calculated cpm and measured cpm was linear with a correlation coefficient $r = 0.981$ for spots from 4 to 955 cpm. The sensitivity of this system allows detection and quantitation of individual spots of less than 5 cpm in radiofluorograms exposed for 96 h.

Results

Growth Hormone Domain of Rat Hepatic mRNA. One of the well-established actions of T_3 is on the pituitary production of growth hormone (Solomon & Greep, 1959; Hervas et al., 1975). Hypothyroid rats are growth hormone deficient, as well as thyroid hormone deficient. Administration of T_3 to such animals will lead to the reappearance of plasma growth hormone. Since the liver is a target organ for both growth hormone and thyroid hormone, it is conceivable that many, if not all, of the T_3-induced changes we have observed in hepatic RNA are actually responses to growth hormone. To test this possibility, we injected hypothyroid animals for 12 days with a dose of ovine growth hormone (0.2 IU per 100 g body weight per day) known to restore the normal growth rate of hypophysectomized rats (Simat et al., 1980). Hepatic RNA extracted from these growth hormone treated hypothyroid animals was compared to RNA from hypothyroid controls or hypothyroid rats which had received 0.3 μg of T_3 per 100 g body weight per day for 12 days—a dose of T_3 estimated to normalize most T_3-responsive parameters including pituitary growth hormone production. The ^{35}S-labeled translational products of these three mRNA populations were subjected to two-dimensional gel electrophoresis, and typical fluorograms of these gels are shown in Figure 1. Since an equal amount of radioactive material (200 000 cpm) was loaded on each gel, any change in the intensity of an individual spot is assumed to represent a change in the relative percentage of the mRNA coding for that product in the total mRNA. Eight translational products were found to be consistently altered in each of four individual rats treated with growth hormone. These products are circled in Figure 1 and numbered in accordance with our previous work (Seelig et al., 1981). This numbering system is not meant to signify any formal nomenclature and is being used for convenience only until the polypeptide corresponding to any spot is identified.

In order to more accurately assess the effects of growth hormone, or other factors, on the genomic expression of rat liver, it was necessary to quantitate individual mRNA levels. Accordingly, we have utilized a system for digitizing video images of fluorograms (Mariash et al., 1982). Computerized digitization of the video image allows for identification of individual "spots". The digitized intensity of any spot can be

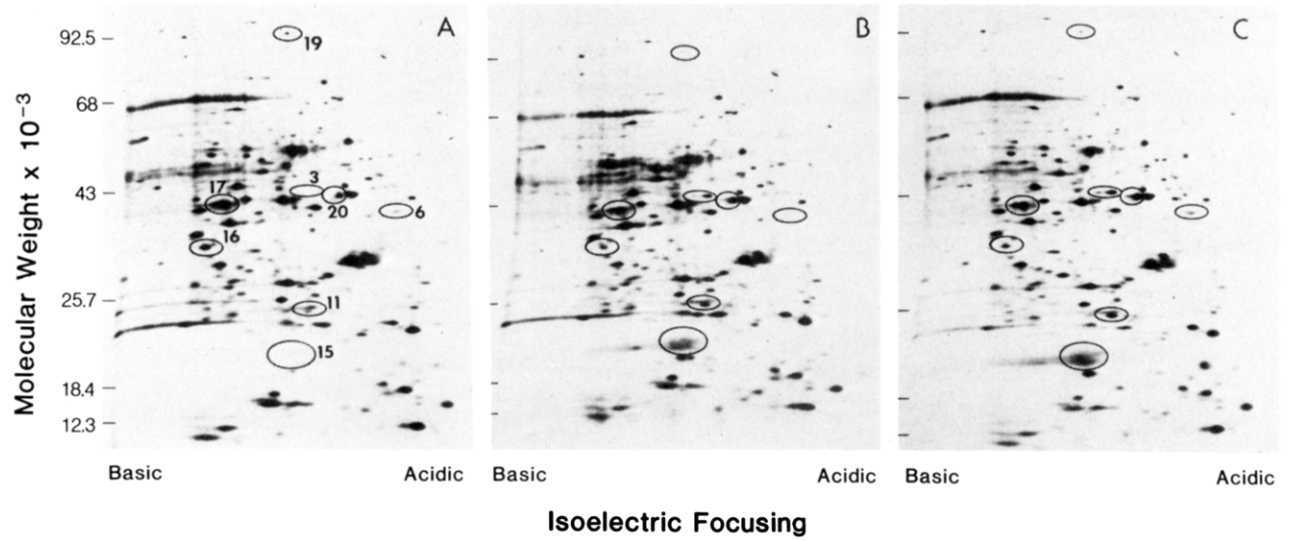

FIGURE 1: Growth hormone domain of rat liver mRNA. Groups of four thyroidectomized rats were treated with vehicle alone (A), 0.2 IU of ovine growth hormone per 100 g body weight per day (B), or 0.3 μg of T_3 per 100 g body weight per day (C) for 12 days. Hepatic poly(A)-containing RNA was extracted and analyzed as described under Experimental Procedures. Fluorographs of representative gels from each experimental group are shown. Only those products which responded reproducibly to growth hormone in each of the four treated animals are circled, and these spots are numbered according to Seelig et al. (1981). Spot 20 is a product not previously detected as T_3 responsive due to overexposure in this area of the gel in earlier work. The pH range of the isoelectric focusing was from approximately 7.9 (basic) to 4.5 (acidic). An equal number of cpm (200 000) was loaded into each gel.

Table I: Growth Hormone Domain of Rat Liver mRNA[a]

spot	Tx control	Tx + growth hormone	Tx + T_3
3	ND	76 (±12)*	160 (±6)*
6	32 (±8)	8 (±3)*	50 (±5)
11	90 (±23)	+	+
15	ND	+	+
16	263 (±48)	121 (±29)*	84 (±16)*
17	1031 (±76)	622 (±51)*	543 (±32)*
19	29 (±7)	8 (±1)*	12 (±7)
20	109 (±13)	273 (±39)*	346 (±5)*

[a] Quantitation of translational products which responded to growth hormone injection is shown. See legend to Figure 1 for experimental details. Tx designates thyroidectomized. Values represent mean cpm (±SEM) based on a total load of 200 000 cpm per gel. A "+" indicates a positive response which could not be quantitated due either to incomplete resolution (spot 11) or to the diffuseness of the spot (spot 15). Values marked with an asterisk are significantly different ($p < 0.05$) from thyroidectomized controls. ND = not detectable.

converted to cpm by using ^{35}S-labeled standards of known cpm density during exposure to the film. The results of such quantitation for the mRNA products by growth hormone administration are shown in Table I.

Two patterns of response to growth hormone can be discerned. In the first type, the direction and magnitude of the response to growth hormone administration are essentially the same as that observed following T_3 treatment. This pattern is seen for spots 16, 17, 19, and 20 and from visual inspection is also probably true for spots 11 and 15, although these latter two products could not be quantitated in these gels due to technical problems. For products in this class, therefore, the entire response observed following T_3 treatment of thyroidectomized animals is compatible with an indirect effect resulting from the well-known T_3 stimulation of pituitary growth hormone production. The second response pattern is one which indicates an interaction between growth hormone and T_3 in determining the normal cellular mRNA concentration. The mRNA species corresponding to spot 3 is only partially restored to normal by growth hormone treatment. Thus, both growth hormone and T_3 are probably necessary for maintenance of this gene product. The response of spot 6 appeared paradoxical in that growth hormone injection attenuated the level, whereas T_3 injection augmented it. It is possible that the T_3 effect on spot 6 represents a direct stimulation of T_3 overriding a simultaneous inhibition by the growth hormone.

Effect of Lipogenic Diet on Hepatic mRNA. Several enzymes involved in lipogenesis can be induced either by feeding of a diet high in simple carbohydrate and fat free or by administration of thyroid hormone (Mariash et al., 1980). For malic enzyme (Towle et al., 1980), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Miksicek & Towle, 1982), the induction by both dietary and hormonal factors is due to an increased intracellular concentration of mRNA coding for these enzymes. Considering the overlap of T_3 and CHO diet in regulating these lipogenic enzymes, we were interested in exploring whether other T_3-responsive mRNA species might also be influenced by the CHO diet.

Groups of hypothyroid, euthyroid, or hyperthyroid rats were fed either the standard laboratory chow diet or the CHO diet for a period of 10–12 days. Hepatic RNA populations extracted from these animals were subsequently analyzed by translation and two-dimensional gel electrophoresis. The overlap found between products responding to CHO diet and to T_3 was quite extensive. For example, the "CHO diet domain" for hypothyroid animals is shown in Figure 2. Ten mRNA translational products were found to be altered by feeding the CHO diet to the hypothyroid rats. Of these, nine spots had previously been identified as T_3 responsive. Only one product was found which was responsive to CHO diet which was not also responsive to T_3. This product, designated spot 22, had an M_r of 58 000 and an approximate p*I* of 6.7.

Examination of dietary effects on euthyroid and hyperthyroid rats also revealed this high degree of overlap (see Table II for quantitation of several products). In each state, spot 22 was the only product responsive to CHO diet which had not been previously designated as a member of the "T_3 domain". The magnitude of the increase measured for spot 22 following feeding of CHO diet was roughly the same in each of the three thyroidal states, indicating no effect of T_3 on this apparent induction (Table II). For the remainder of

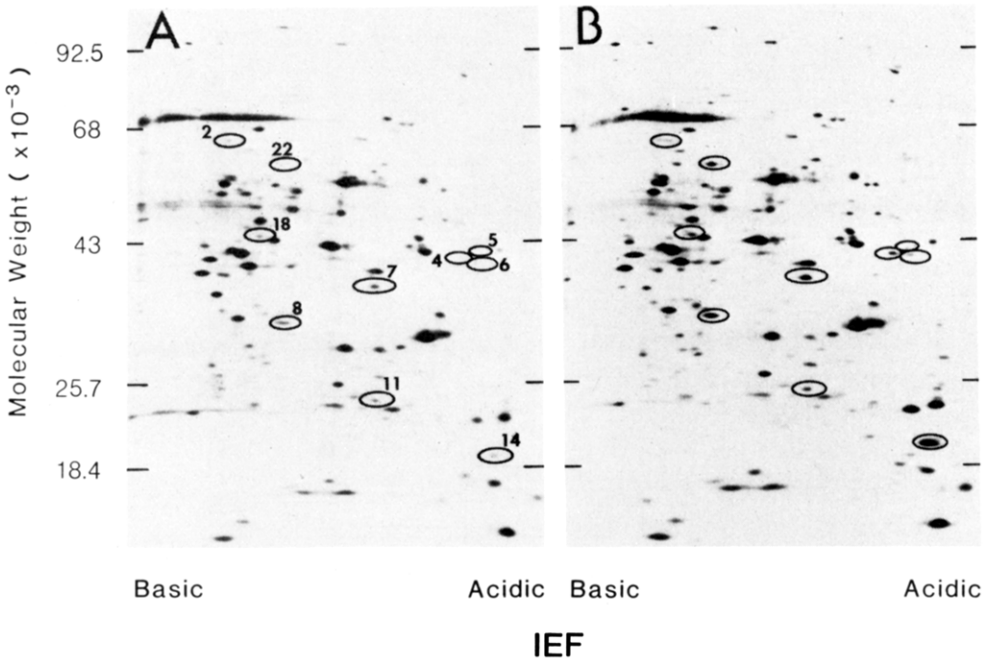

FIGURE 2: Comparison of representative two-dimensional electrophoretograms of hepatic mRNA directed translational products from thyroidectomized rats fed either standard chow diet (A) or CHO diet (B). Animals were maintained on diets ad libitum for a period of 10 days. Spots circled and numbered are those that respond to the CHO diet in this thyroid state. Spot 22 was not previously designated, as it is not responsive to T_3 in chow-fed rats.

Table II: Quantitation of Several Representative T_3- and CHO-Responsive mRNA Products[a]

	Tx		Eu		Hyper	
spot	chow	CHO	chow	CHO	chow	CHO
3	ND	ND	197 (±3)	130 (±4)	301 (±25)	378 (±167)
5	ND	15 (±2)	55 (±10)	90 (±16)	86 (±9)	177 (±13)
7	44 (±12)	119 (±21)	24 (±5)	50 (±6)	250 (±33)	153 (±14)
10	274 (±76)	302 (±51)	226 (±40)	23 (±17)	ND	32 (±32)
11	10 (±8)	42 (±9)	81 (±17)	279 (±71)	609 (±51)	571 (±71)
14	20 (±11)	412 (±37)	204 (±20)	515 (±21)	576 (±23)	894 (±113)
17	700 (±71)	766 (±37)	316 (±30)	273 (±25)	316 (±100)	240 (±56)
22	12 (±1)	101 (±6)	17 (±1)	68 (±6)	5 (±2)	125 (±27)

[a] Thyroidectomized (Tx), euthyroid (Eu), or hyperthyroid (Hyper) (15 μg of T_3 per 100 g body weight per day) rats were maintained on either standard chow diet or CHO diet for a period of 10 days before analysis. Values represent mean cpm (±SEM) based on a total load of 200 000 cpm per gel. ND = not detectable.

the responsive products, the direction of the change (i.e., positive or negative) was the same for the switch to the CHO diet as for increasing T_3 levels. Thus, spot 14 is increased by both T_3 and CHO diet, whereas spot 10 is decreased by both.

Closer examination of several of the mRNA sequences which exist in the T_3 and CHO diet domains allows us to differentiate three patterns of interaction (Table II, Figure 3). Monoresponsive mRNA species are those which respond to one or the other stimuli, but not to both. Examples are spot 22, which responds to CHO diet but not T_3, and spot 3, for which the response to T_3 is the same on both diets. A second pattern is seen for those translational products in which T_3 and CHO diet both affect the mRNA species (biresponsive) in a noncooperative fashion. This pattern is illustrated by spot 14 and is indicated by the parallel nature of the T_3 response curves for animals on regular and CHO diets. The magnitude of the response for the two stimuli together is largely additive of the individual responses to each alone. The third type of response pattern is also biresponsive, but in a cooperative fashion. For these mRNA sequences, an interaction is indicated by the nonparallel nature of the T_3 response curves on the two diets. Both positive (spot 11) and negative (spot 10) cooperativities have been noted. Thus, as seen earlier with growth hormone, a complex response pattern indicating varying degrees of interaction between CHO diet and T_3 is noted for several responsive mRNA species in the T_3 domain.

Time Course of T_3 Response. The studies to this point have examined effects of T_3 at approximate steady-state conditions. We were interested in studying the time course of these responses to determine whether any changes appear rapidly after T_3 administration. For this purpose, hypothyroid rats were injected with a single dose of 200 μg of T_3—a dose designed to saturate the nuclear receptor sites throughout the experiment (Oppenheimer et al., 1977). At various times after injection, poly(A)-containing RNA was extracted from the livers of these animals and analyzed. The time course of response of the various products in the T_3 domain was highly diverse. Several representative responses are shown in Figure 4, and quantitation of these products is given in Table III. At 4 h following T_3 injection, two products were found to be significantly changed. Spot 6 had undergone about a 2-fold increase; spot 14 had increased by over 13-fold at this early time point. Examination of an earlier time point, 1.5 h, revealed that spot 14 had increased 4–5-fold in this time interval (data not shown). No other product observed on these two-dimensional gels was demonstrably affected at 1.5 h. Moreover, nonequilibrium two-dimensional gel electrophoresis—a technique designed to examine proteins with p*I*s more basic

Table III: Quantitation of Time Course Data[a]

spot	Tx control (A)	Tx + 4-h T3 (B)	Tx + 8-h T3 (C)	Tx + 12-h T3 (D)	Tx + 12-day T3 (E)
3	ND	ND	ND	ND	378 (±168)*
4	ND	ND	ND	ND	240 (±2)*
5	ND	ND	ND	ND	86 (±9)*
6	42 (±8)	104 (±13)*	137 (±11)*	224 (±14)*	149 (±65)*
7	43 (±5)	54 (±15)	101 (±6)*	102 (±29)*	250 (±33)*
10	350 (±37)	298 (±30)	271 (±22)	199 (±13)*	ND*
14	30 (±8)	397 (±57)*	284 (±55)*	436 (±59)*	576 (±23)*
21	98 (±8)	78 (±11)	73 (±17)	19 (±12)*	8 (±5)*

[a] Thyroidectomized rats were injected with 200 μg of T_3 per 100 g body weight at time zero, and at varying times thereafter, livers were removed for analysis. Hyperthyroid animals (E) received 15 μg of T_3 per 100 g body weight per day. Values represent mean cpm (±SEM) with an n of 4 based on a total load of 200 000 cpm per gel. Values marked with an asterisk are significantly different ($p < 0.05$) from thyroidectomized controls. ND = not detectable.

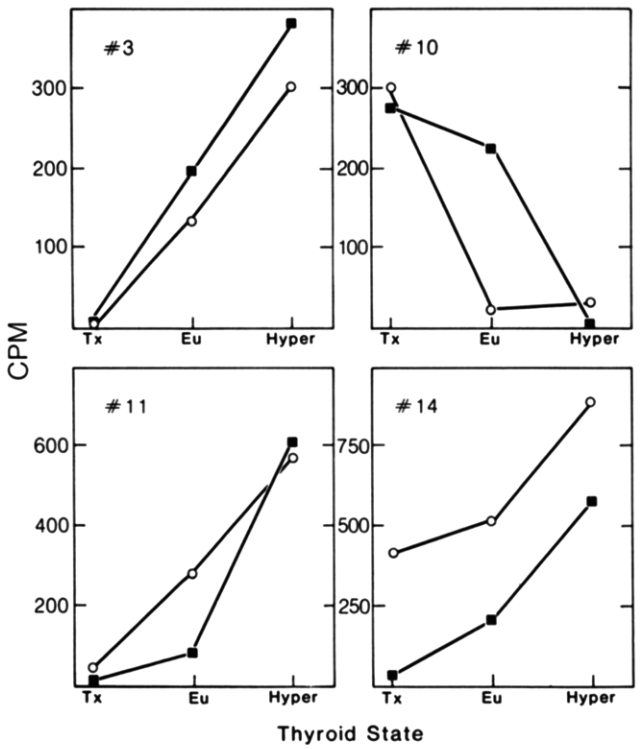

FIGURE 3: T_3 response curves of spots 3, 10, 11, and 14 in animals maintained either on chow diet (■) or CHO diet (○). Euthyroid animals are placed at the midpoint of the abscissa since the estimated T_3 receptor occupancy level of normal animals is 50% (Oppenheimer, 1979).

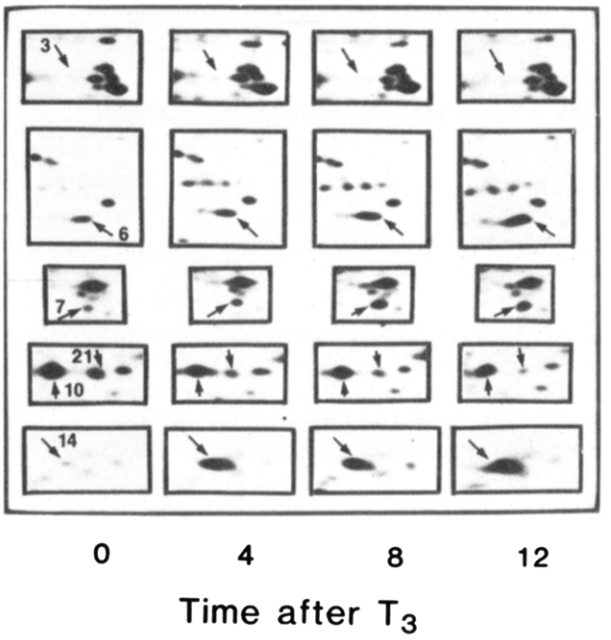

FIGURE 4: Time course of response of several representative mRNA species to T_3. Thyroidectomized rats were injected with a single dose of 200 μg of T_3 at time 0 and killed at varying times after treatment. Hepatic poly(A)-containing RNA from these animals was translated and anaylzed by two-dimensional electrophoresis of the products. For clarity, several T_3-responsive products were selected and cut out of the electrophoretogram. The numbering corresponds to that given in Seelig et al. (1981). For spot 3, which does not appear in the first 12 h following T_3, the arrow points to the expected position of this product.

than 7 (O'Farrell et al., 1977)—also failed to reveal any other differences at this time point. Unfortunately, nothing is known on the nature or function of spot 14, other than its approximate M_r of 18 000 and pI of 4.9. It does, however, represent the earliest mRNA response to T_3 which has been measured to date.

The lag time between administration and response for several of the other T_3-responsive products varied between 4 and 12 h. This time lapse was applicable both for products with augmented levels, such as spot 7, and for products which were attenuated by T_3, such as spot 10. Several other translational products, such as spot 3, did not show any appreciable effects until as late as 24 h following T_3 injection. Such relatively late responses to T_3 treatment probably represent indirect effects of T_3.

T_3 Domain of Rat Kidney. In light of the dramatic effects of T_3 on the hepatic mRNA population, it was of interest to examine another target organ for thyroid hormone to determine the tissue specificity of T_3 responses. For this purpose, we chose the kidney, which has T_3 nuclear receptor levels comparable to those of liver in the rat (Oppenheimer et al., 1974). Due to the higher endogenous levels of ribonuclease in rat kidney, we used a Gdn·HCl extraction method for obtaining intact total kidney RNA, rather than the phenol–chloroform method used for liver RNA extractions (Deeley et al., 1977; Chirgwin et al., 1979). In control experiments, two-dimensional gel patterns of liver RNA extracted by using either of these two methods were almost indistinguishable. All of the hepatic T_3-responsive products detected previously were also found to be responsive in hepatic RNA extracted by the Gdn·HCl method (data not shown). Figure 5 shows representative two-dimensional gel patterns of translational products directed by renal poly(A)-containing RNA of hypothyroid or hyperthyroid animals. At least seven mRNA species in the kidney were found to respond reproducibly to T_3. As seen with hepatic RNA, both positive (spots 1, 3, 4, 5, and 7) and negative (spots 2 and 6) responses were observed with increasing T_3 levels. Whereas the pattern of translational products in kidney and liver are clearly distinct, close examination does reveal many products which coincide between the

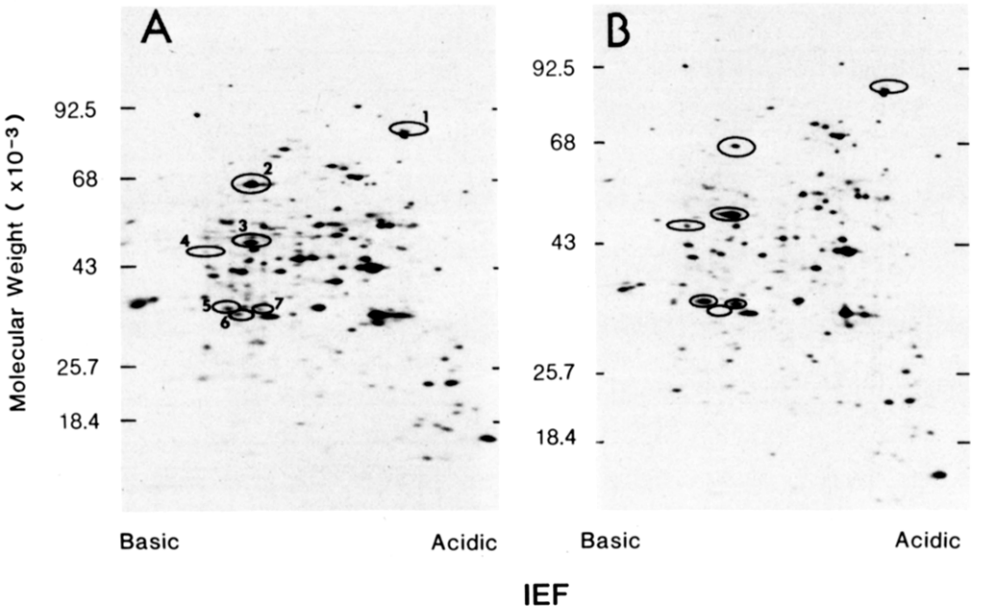

FIGURE 5: Comparison of mRNA-directed translational products from kidneys of hypothyroid or hyperthyroid rats. Thyroidectomized rats were either injected with vehicle (A) or with 15 μg of T_3 per 100 g body weight per day (B) for 10 days. Poly(A)-containing RNA was isolated from the kidneys of these animals as described under Experimental Procedures and analyzed as for hepatic RNA. Products which were found to be altered in all members of the experimental group are circled and numbered arbitrarily.

two RNA populations at least with respect to p*I* and M_r. However, none of the major T_3-responsive mRNA species were found to be in common between the two tissues. Thus, the T_3 domains of these two target organs appear to be largely nonoverlapping at this level of resolution, indicating that each target tissue responds to hormone in a unique and characteristic fashion.

Discussion

In this paper, we have explored the pleiotropic effects of thyroid hormone, growth hormone, and CHO diet on rat liver mRNA. The experimental approach utilized the technique of two-dimensional gel electrophoresis to separate mRNA-directed translational products of total hepatic poly(A)-containing RNA. Approximately 200–250 different mRNA species were examined simultaneously by this method. Treatment of animals with thyroid hormone, growth hormone, or CHO diet each led to significant changes in the levels of a subset of these mRNA species. In accordance with the nomenclature suggested by Tomkins (1975), each subset of mRNA sequences responding to a specific effector was termed a domain of rat liver mRNA. Perhaps the most interesting finding of this work is the high degree of overlap between the domains of T_3 and growth hormone and between the T_3 and CHO diet. Thus, many hepatic mRNAs appear to be regulated in a complex multifactorial fashion. Before the implications of these results are discussed, however, several aspects of the technical approach should be mentioned.

The absolute quantitation of an individual translational product on the two-dimensional gel represents an approximation of the true mRNA level present in liver. Poly(A)-containing RNA as selected by chromatography on oligo(dT)–cellulose was used throughout this study. Thus, certain mRNA species lacking poly(A) tails would not be detected, whereas others might be over- or underrepresented in the poly(A)-containing RNA fraction due to potential variation in poly(A) lengths on any particular mRNA species. In such a case, the absolute level of any particular mRNA would be over- or underestimated; however, a comparison of its relative level between different thyroidal states should be valid assuming T_3 does not influence the partition of the mRNA species between poly(A+) and poly(A−) fractions. We have previously shown that thyroid hormones do not affect the average poly(A) tail length of hepatic mRNA (Towle et al., 1979); however, the possibility of such an influence on any individual mRNA cannot be ruled out. Similar considerations are also warranted with regard to the translational efficiency of individual mRNA species in the reticulocyte lysate system. While in most cases one would expect an mRNA species to be translated in proportion to its abundance in the total mRNA population, variations in translational efficiency of individual mRNAs are well documented [e.g., see Palmiter (1974), Sonenshein & Brawerman (1977), and Asselbergs et al. (1980)]. A final caution with regard to quantitation of individual mRNA levels regards the use of [^{35}S]methionine for detection of translational products. Any polypeptide which has a higher or lower methionine content than the average content of all liver proteins will be proportionally over- or underestimated. Despite these limitations, it is likely that in most cases the quantitation of any individual mRNA level will be a reasonable reflection of its concentration in the total mRNA. Furthermore, comparisons of the levels of any translational product between mRNA populations isolated from different animals should be a valid means of assessing relative changes in that particular mRNA.

Several other aspects of the two-dimensional electrophoretic technique for analyzing mRNA translational products are noteworthy. The number of different products which we routinely visualize is in the range of 150–250, depending on exposure times. Obviously, this represents only a small fraction of the total complexity of rat liver mRNA, which is variously estimated between 11 000 and 23 000 different sequences (Coupar et al., 1978; Savage et al., 1978; Towle et al., 1979). Some translational products will have p*I*s beyond the range of the isoelectric gradient employed, approximately 4.8–7.5. The majority of products which do fall in the gradient, however, remain undetected, presumably because the concentration of these mRNA species is a very small percentage of the total mRNA. Analyses of cDNA–mRNA hybridization kinetics have led to the finding that rat liver mRNA can be divided

into three general groups on the basis of relative mRNA concentration (Coupar et al., 1978; Savage et al., 1978; Towle et al., 1979). The most highly abundant products represent about 25% of the total mRNA but consist of only six to eight different mRNA species. An intermediate abundancy class contains approximately 35% of the total mRNA molecules and 300–350 different mRNA sequences. The translational products detected by two-dimensional gel analysis thus represent members of these two abundancy classes of mRNA. The majority of the sequence complexity of rat liver mRNA is contained in the remaining 40% of mRNA molecules which is composed of species present in only a few copies per cell. The level of any individual mRNA sequence in this class is too low in concentration to be detected considering the number of cpm loaded and the duration of fluorography used in this study. Whether the results observed for mRNA sequences present in the high and intermediate abundancy classes detected here are indicative of the low abundancy mRNA species is a matter of conjecture.

A final noteworthy point regarding the techniques is the presence of what we have termed "variable" products. These are spots which changed in intensity substantially from animal to animal even within a single experimental group. Several such products were observed. One possibility is that these variable spots are responding to unknown factors of genetic, environmental, or hormonal nature which we have not been able to sufficiently control. A translational product was designated responsive to any treatment only if it was affected in all individual animals in the experimental group of at least four.

Several other workers have utilized the powerful resolution of the two-dimensional gel technique to analyze the pleiotropic effects of glucocorticoid hormones or cAMP levels on the rate of synthesis of specific proteins in cultured cells (Alton & Lodish, 1977; Ivarie & O'Farrell, 1978; Garrels & Schubert, 1979). In these studies, cells were labeled for short periods of time with [^{35}S]methionine, and total cell protein was extracted and subjected to two-dimensional electrophoresis. This approach should encompass potential hormonal actions on both mRNA levels and translational efficiency of individual mRNA species. In our studies, we have extracted mRNA and translated in a cell-free protein synthetic system. This approach provides two advantages. First, in whole animal studies, pulse labeling of proteins with radioactive amino acids is far less efficient than in cultured cells. The lower specific activity of protein obtained in vivo makes it technically difficult to analyze by using two-dimensional gel electrophoresis. Second, we should be more directly assessing the effects of hormone at the pretranslational level.

Recently Ivarie et al. (1981) have examined the action of thyroid and glucocorticoid hormones in altering the rate of synthesis of proteins in cultured rat pituitary tumor cells. Fifteen proteins were found to be affected by T_3, 10 of which were increased and 5 decreased. Since the authors estimated that 1000 different spots were detectable, about 1–2% of the proteins were in the T_3 domain in this cell line. The action of T_3 in hepatic mRNA levels appears to be somewhat more widespread. We have estimated that approximately 8% of the translational products are altered by T_3.

The time course of response to T_3 was highly diverse for various mRNA species in the thyroid hormone domain. The mRNA corresponding to spot 14 increased 4–5-fold by 1.5 h, the earliest time point measured. Several other products such as spots 3 and 4 showed no detectable change within 12 h of T_3 treatment. These findings argue against a simple, single class of T_3-responsive gene products, and for a cascade-type mechanism in which early T_3 effects on certain gene products lead indirectly to later effects on other genes. An example of an indirect action of T_3 for certain members of the T_3 domain is demonstrated by the presence of growth hormone responsive products. When growth hormone was injected into hypothyroid rats, eight translational products were found to respond. In all but one case (spot 6), the responses were in the same direction (positive or negative) as that seen with T_3 injection. The action of T_3 on these mRNA species, therefore, may potentially be mediated by the well-documented stimulation of growth hormone production by T_3 (Tsai & Samuels, 1974; Hervas et al., 1975). Consistent with this interpretation is the observation that none of these growth hormone responsive products is affected at the earlier time points examined after T_3 administration to hypothyroid rats. The lag time might be due in part to the time required for T_3 to stimulate growth hormone production to effective levels. However, it should be pointed out that all preparations of growth hormone extracted from whole pituitaries are likely contaminated with other hormones (see Experimental Procedures). Thus, it is impossible to conclude unambiguously that growth hormone is the active principle for each of the mRNA species which responds to this preparation. In addition, it is possible that an action of growth hormone itself could be indirectly occurring through stimulation of somatomedin levels or other unknown factors. Further experiments using growth hormone prepared from the cloned growth hormone gene and isolated hepatocytes will be necessary to address these issues. For the remainder of this discussion, it will be assumed that growth hormone is the mediator of action in the preparation.

Two general response patterns to growth hormone can be distinguished. For some, the administration of growth hormone led to virtually the same level of response as that observed following T_3 administration. Spots 11, 17, 19, and 20 were members of this group. For these mRNA species, it appears that growth hormone is solely responsible for regulation, and they would more appropriately be designated as members solely of the growth hormone domain of rat. On the other hand, spot 3 was only partially restored to normal levels by growth hormone treatment, whereas spot 6 responded in opposite directions to T_3 and growth hormone. These mRNA species must require some other action of T_3 other than its effect on growth hormone production for normal expression. These gene products thus are members of overlapping domains between two hormones capable of acting on liver. Examples of such multihormonal regulation of specific gene products involving T_3 have been elucidated previously: α_{2u}-globulin requires T_3, growth hormone, testosterone, and glucocorticoid for normal levels to be maintained (Roy, 1973; Kurtz & Feigelson, 1978) whereas expression of the gene for growth hormone is affected by both T_3 and glucocorticoid (Samuels et al., 1977; Martial et al., 1977b).

The ability of growth hormone administration to alter specific mRNA levels has been documented in only two cases. Keller & Taylor (1979) have found hepatic albumin mRNA levels to be decreased approximately 50% following hypophysectomy of rats and restored to near normal by growth hormone treatment. Roy & Dowbenko (1977) reported that growth hormone treatment of hypophysectomized rats which were simultaneously given dihydrotestosterone, corticosterone, and thyroxine led to elevated levels of mRNA for α_{2u}-globulin. By the techniques used in this paper, we find several mRNA species in rat liver responsive to growth hormone. It is interesting that growth hormone, like T_3, can both increase (e.g.,

spot 3) and decrease (e.g., spot 17) specific mRNA levels. Thus, this hormone which is thought to act through a receptor located in the cell membrane (Ranke et al., 1976; Turyn & Dellacha, 1978) can act at a pretranslational level to affect specific protein synthesis.

In addition to the interactions of growth hormone and T_3 in regulating hepatic mRNA levels, we were also interested in studying the effects of high carbohydrate, fat-free diets. Switching animals from chow diet to CHO diet is known to induce a set of enzymes involved in lipogenesis. In several cases, elevated mRNA levels for specific enzymes have been demonstrated to accompany this induction (Hutchinson & Holten, 1978; Flick et al., 1978; Nepokroeff & Porter, 1978; Cladaras & Cottam, 1980). Many of these lipogenic enzymes are also responsive to T_3, although the degree of response varies from enzyme to enzyme (Mariash et al., 1980). For the mRNA species detected by two-dimensional electrophoresis, we also see a high degree of overlap between the thyroid hormone domain and the CHO diet domain. We find only one translational product (spot 22) in hepatic mRNA which responds to the CHO diet and which was not previously identified as a member of the T_3 domain. On the other hand, many translational products (spots 2, 4, 5, 6, 7, 8, 10, 11, 14, and 21) of the T_3 domain are also influenced by CHO diet. One possibility to explain the overlapping domains of T_3 and CHO diet is that both treatments are stimulating a common cellular signal responsible for the influence on various mRNA species. This scheme does not seem likely. First, the presence of products which are exclusively in one domain or the other (e.g., spot 9 for T_3 or spot 22 for CHO diet) cannot be explained by a common regulatory signal generated by both treatments. Second, the presence of both noncooperative and cooperative patterns of interaction between T_3 and CHO diet does not fit with this simple notion. For those mRNAs which display noncooperative interaction, the signals generated by CHO diet and T_3 would appear to be acting independently of each other. For the majority of the mRNA species influenced by T_3 and CHO diet, a cooperative influence of the two treatments was observed. In these cases, it is reasonable to assume that the two regulatory pathways stimulated by T_3 and CHO diet must interact at some step in the production of mRNA.

Added in Proof

We have recently identified spot 2, a product that responds to both T_3 and CHO diet, as malic enzyme by specific immunoprecipitation of translational products followed by two-dimensional gel electrophoresis. Previous work has shown that the mRNA coding for malic enzyme responds to both of these stimuli (Towle et al., 1980).

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Registry No. T_3, 6893-02-3; growth hormone, 9002-72-6.

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Tubulin–Zinc Interactions: Binding and Polymerization Studies†

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ABSTRACT: The binding of Zn^{2+} to tubulin and the ability of this cation to promote the polymorphic assembly of the protein were examined. Equilibrium binding showed the existence of more than 60 potential Zn^{2+} binding sites on the dimer, including a number of high-affinity sites. The number of high-affinity sites, estimated by using a standard amount of phosphocellulose to remove more weakly bound Zn^{2+}, reached a maximum of 6–7.5 with increasing levels of Zn^{2+} in the incubation solution. The number also increased with time of incubation at a single Zn^{2+} concentration. It is suggested that tubulin is slowly denatured in the presence of Zn^{2+}, exposing more binding sites. Cu^{+} and Cd^{2+} were effective inhibitors of Zn^{2+} binding; Mg^{2+}, Mn^{2+}, and Co^{2+} were much less effective, and Ca^{2+} was without effect. Zn^{2+} does not replace the tightly bound Mg^{2+}. GTP reduces the amount of Zn^{2+} binding under equilibrium conditions and the amount bound to high-affinity sites. Zinc-induced protofilament sheets are produced at a Zn^{2+}/tubulin ratio of 5 in the presence of 0.5 mM GTP, conditions where about two to three Zn^{2+} ions would be bound to the dimer. At higher GTP concentrations, less assembly occurred, and the products were narrower sheets and microtubules. Zn^{2+}–tubulin, isolated from phosphocellulose, will not assemble unless Mg^{2+} and dimethyl sulfoxide (Me_2SO) or more Zn^{2+} is added. Broad protofilament sheets, formed from Zn^{2+}–tubulin in the presence of Mg^{2+} and Me_2SO, contain slightly more than one Zn^{2+} per dimer. It is concluded that Zn^{2+} stimulates tubulin assembly by binding directly to the protein, not via a ZnGTP complex.

Assembly of the tubulin dimer into protofilamentous structures is stimulated by a variety of agents, including microtubule-associated proteins, organic solvents, and divalent metal ions. Stimulation by divalent cations can lead to microtubules or a variety of polymorphic structures. Microtubules are usually produced in the presence of Mg^{2+} (Lee & Timasheff, 1975; Herzog & Weber, 1977) and Mn^{2+} (Buttlaire et al., 1980), although at high concentrations of Mg^{2+} double rings (Frigon & Timasheff, 1975), latticelike arrays of rings (Voter & Erickson, 1979; Zabrecky & Cole, 1980; Haskins, 1981), ribbons, and multiple tubule structures (Carlier & Pantaloni, 1978; Haskins, 1981) are also produced.

Co^{2+} and Zn^{2+} can also promote the assembly of microtubules (Haskins et al., 1980; Gaskin, 1981) but more generally lead to the formation of several types of polymorphic structures (Larsson et al., 1976; Wallin et al., 1977; Gaskin & Kress, 1977; Gaskin, 1981; Haskins et al., 1980). The major structural forms produced in the presence of these two cations are sheets or ribbons containing variable numbers of protofilaments. The structure of sheets produced by Zn^{2+} has been examined by image reconstruction, and the sheets apparently are constructed from protofilaments arranged in an alternating antiparallel fashion (Baker & Amos, 1978; Tamm et al., 1979). Structures morphologically similar to the Zn^{2+} sheets have been observed in both Zn^{2+}-treated cultures of dorsal root ganglion (Gaskin et al., 1978) and swollen dendrites, when zinc wires were implanted into the brains of rats (Kress et al., 1981).

At present, information pertaining to the number of Zn^{2+} binding sites on tubulin is not available. The purpose of this study was to examine the binding of Zn^{2+} to tubulin and relate this binding to the ability of Zn^{2+} to promote the polymorphic assembly of tubulin.

Experimental Procedures

Materials. GTP, EGTA, Pipes, and Mes were purchased from Sigma Chemical Co. In most experiments, buffer and GTP solutions were passed through phosphocellulose columns to remove divalent cations. $^{65}ZnCl_2$ was a product of New England Nuclear Corp.

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