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Article de synthèse

Structure and regulation of the avian gene for fatty acid synthase

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Summary — Starvation, glucagon and cyclic AMP inhibit, and refeeding starved animals and insulin or IGF-I plus triiodothyronine stimulate accumulation of FAS and its mRNA in liver; transcription is the primary regulated step. In the uropygial gland, differentiation of basal cells into mature sebocytes is accompanied by the accumulation of large amounts of FAS and its mRNA. By analogy with liver, transcription is likely to be the regulated step, but direct experimental evidence for this hypothesis is lacking. FAS mRNA is a unique gene and is probaby more than 100 kb in length. The FAS gene of goose and duck is transcribed into two mature mRNAs of about 10 800 and 12 200 nucleotides. The 3'-untranslated regions of the FAS mRNAs contain an unusual polypyrimidine tract which, at the mRNA level at least, appears unrelated to regulation of gene expression. Polypyrimidine tracts similar in sequence to that in the FAS gene are found in about 20 different parts of the genome. All of the fragments which contain these tracts are hypermethylated. The next stage of this investigation will involve identification of *cis*-acting sequence elements in the FAS gene which specify responses to diet, hormones and tissue-specific regulatory factors. Isolation and characterization of the 5'-ends of the cDNA and the gene are underway.

fatty acid synthase — mRNA — birds

Résumé — Structure et régulation du gène de l'acide gras synthétase des oiseaux. *Le glucagon et l'AMP cyclique, tout comme le jeûne, inhibent l'accumulation de l'acide gras synthétase (AGS) et de son ARNm dans le foie. La réalimentation des animaux préalablement mis à jeun et l'insuline ou l'IGF-1 associée à la triiodothyronine ont des effets inverses. L'étape primaire de la régulation se situe au niveau transcriptionnel. Dans la glande uropygiale, la différenciation des cellules basales en sébocytes matures est également associée à l'accumulation d'une importante quantité d'AGS et de ARNm correspondant. Par analogie avec ce qui est observé au niveau hépatique, la régulation intervient probablement au niveau transcriptionnel, mais la démonstration directe de cette hypothèse n'a pas encore été apportée. Le gène codant pour l'AGS est un gène unique d'une longueur probablement supérieure à 100 kb. Il est transcrit chez l'oie et le canard en deux ARNm matures d'environ 10 800 et 12 200 nucléotides. Les régions 3' des ARNm contiennent*

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un inhabituel enchaînement de type polypyrimidine qui, au moins au niveau du ARNm, ne semble pas associé à la régulation de l'expression du gène. Des fragments polypyrimidine similaires à ce qui est observé dans le gène AGS ont été mis en évidence dans environ 20 parties différentes du génome. Tous les fragments contenant ces enchaînements sont hyperméthylés. La prochaine étape de ce travail comportera l'identification des éléments de la séquence qui, dans le gène de l'AGS, contrôlent les réponses à l'alimentation, aux hormones et aux facteurs de régulation tissulaire. L'isolement et la caractérisation des parties 5' du ADNc et du gène sont en cours.

acide gras synthétase — gène codant — oiseaux

Introduction

Avian fatty acid synthase (FAS) is a multifunctional polypeptide which catalyzes the synthesis of palmitate from acetyl-CoA, malonyl-CoA, and NADPH (Wakil *et al.*, 1983). It is one of a set of hepatic 'lipogenic' enzymes, whose activities are inhibited by starvation and stimulated by refeeding starved animals (Wakil *et al.*, 1985; Goodridge, 1985, 1986). In maintenance cultures of chick embryo hepatocytes, FAS activity is stimulated by insulin and thyroid hormone and inhibited by glucagon and cyclic AMP (Goodridge *et al.*, 1974; Fischer and Goodridge, 1978). *In vivo,* it is probable that increased blood concentrations of insulin and triiodothyronine and decreased concentration of glucagon are important in establishing the high levels of enzyme characteristic of the fed state; the opposite is true for the starved state (Goodridge *et al.*, 1986). We and others have used immunological techniques to establish that these changes in enzyme activity are due to comparable changes in the concentration of FAS rather than to changes in catalytic efficiency of the enzyme; the changes in enzyme concentration, in turn, are due to selective regulation of the synthesis of FAS as opposed to regulation of enzyme degradation (Zehner *et al.*, 1977; Fisher and Goodridge, 1978).

Enzyme synthesis can be controlled by regulating the amount of the relevant mRNA or by regulating the efficiency with which a constant amount of that mRNA is translated into protein. Analysis of the amount and turnover of FAS mRNA requires a reagent which specifically recognizes FAS mRNA. This reagent should bind to and allow the selective recovery of FAS mRNA. A complementary DNA (cDNA) is such a reagent and was obtained by molecular cloning techniques.

Isolation of FAS cDNAs and characterization of the mRNA

Isolating cloned cDNAs is much simpler if the relevant mRNA is abundant. FAS represents 20—30% of the mass of total protein in the uropygial gland of the domestic goose (Buckner and Kolattukudy, 1976), a level much higher than that found in chicken liver (Fischer and Goodridge, 1978). The uropygial gland enzyme is physically, kinetically and immunologically identical to the liver enzyme (Buckner and Kolattukudy, 1976), suggesting that they are products of the same gene. Furthermore, antisera to goose uropygial gland FAS cross-reacts strongly with the chicken liver enzyme (Buckner and Kolattukudy, 1976; Goodridge, unpublished results), indicating that the amino acid sequences

of the chicken and goose enzymes are very similar and, therefore, that the nucleotide sequences for FAS mRNAs of the two species would be sufficiently similar to allow cross-hybridization. After preparing a cDNA library from total mRNA extracted from the goose uropygial gland, bacterial colonies containing FAS cDNAs were identified by hybridization with a radiolabeled cDNA copied from partially purified FAS mRNA (Morris *et al.*, 1982). Several positive clones were isolated and shown to contain FAS sequences by hybrid-selected translation (Morris *et al.*, 1982).

The initial clones represented about 2 600 nucleotides from the 3'-untranslated region of FAS mRNA. These cloned DNAs were used as probes to demonstrate the presence of two FAS mRNAs in the livers and uropygial glands of geese and ducks and in the livers of chickens. The goose and duck mRNAs are about 10 800 and 12 200 nucleotides in length (Fig. 1); the chicken mRNAs are about 10 000 and 9400 nucleotides (data not illustrated). Both the long and short mRNAs are transcribed from a unique FAS gene. A combination of : 1) comparison of restriction fragment lengths in genomic DNA and cloned FAS cDNAs; 2) differential hybridization of cloned cDNAs to the two mRNAs; and 3) nucleotide sequence analysis indicates that the longer mRNA is a 3'-extension of the shorter one.

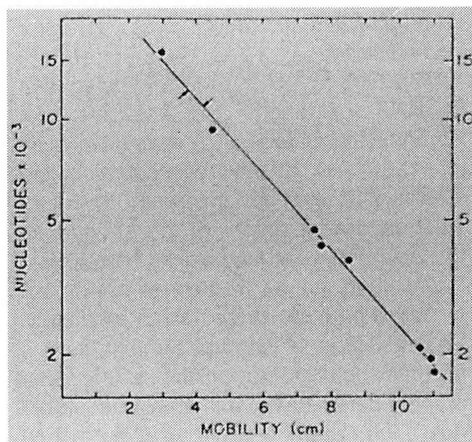

Fig. 1. Determination of the size of FAS mRNAs by Northern blot analysis. Total RNA was extracted from the livers or uropygial glands of fed goslings and subjected to electrophoresis in 0.8% agarose gels containing 2.2 M formaldehyde. The gel and electrophoresis buffers consisted of 20 mM 4-morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0. The separated RNAs were blot-transferred onto nitrocellulose paper and hybridized to ^{32}P-labeled cDNA copied from M13GFAS3Bm (Back *et al.*, 1986). Left panel : gosling liver, 3 μg (lane 1); gosling uropygial gland, 3 μg (lane 2), duck liver, 3 μg (lane 3), and chicken liver, 10 μg (lane 4). Right panel, plot of the migration of marker RNAs, with the positions of FAS mRNAs indicated by the arrows. The molecular weight markers were silk moth fibroin mRNA (16 000 nucleotides), Rous sarcoma virus genomic RNA (9 300 nucleotides), mouse 28S ribosomal RNA (4 700 nucleotides), chicken 27S ribosomal RNA (4 250 nucleotides), duck 27S ribosomal RNA (3 820 nucleotides), silk moth 18S ribosomal RNA (2 100 nucleotides), mouse 18S ribosomal RNA (1 930 nucleotides), and duck 18S ribosomal RNA (1 780 nucleotides). Modified from Back *et al.* (1986) and to which the reader should refer for additional information.

Nutritional regulation of the level of FAS mRNA

The relationship between the relative rate of synthesis of FAS and abundance of its mRNA was investigated in chicks and ducklings prior to and immediately following hatching. Synthesis of hepatic FAS increased about 100-fold between the 19th day of incubation and 4 days after hatching in the livers of unfed chicks. Feeding the newly-hatched chicks caused hepatic enzyme synthesis to increase to 6

times the rate in unfed chicks (Fischer and Goodridge, 1978; Zehner *et al.*, 1977). FAS mRNA was hardly detectable in RNA extracted from the livers of embryos incubated for 16 days (Morris *et al.*, 1984). By 19 days of incubation, the FAS mRNA level had increased by 5-fold. At 2 days after hatching, the abundance of FAS mRNA in unfed chicks was 30-fold higher than that at 16 days of incubation. If neonatal birds were fed immediately, FAS mRNA level increased to 5 times that in unfed chicks. In chicks which were starved for 2 days and then refed for 1 day, the abundance of FAS mRNA increased more than 6-fold. In the reciprocal experiment, mRNA level decreased by 90% when fed newly hatched chicks were starved (Morris *et al.*, 1984). Thus a strong positive correlation between the rate of synthesis of FAS and the level of its mRNA was observed during the increase in hepatic FAS which occurred during the hatching period and was independent of the nutritional state, as well as during changes in enzyme level caused by feeding and starvation (Goodridge, 1973). We conclude that regulation of FAS activity is exerted primarily at a pretranslational step. A similar set of observations was made using perinatal ducklings (Goodridge *et al.*, 1984).

Mechanisms which regulate abundance of FAS mRNA in liver

The concentration of a specific mRNA is a function of its rates of both production and degradation. We estimated the half-life of FAS mRNA in fed and starved ducklings by determining the rate at which the level of FAS approached a new steady state after refeeding starved ducklings or starving fed ducklings (Back *et al.*, 1986).

FAS mRNA accumulated rapidly in the liver when starved neonatal ducklings were fed (Fig. 2). By 9 h after initiation of feeding, the level had increased about 10-

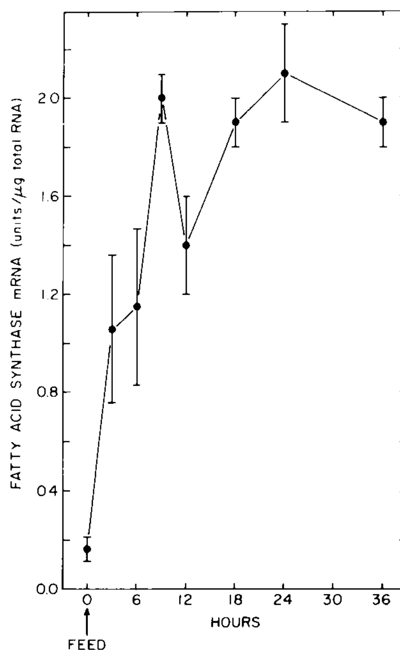

Fig. 2. The effect of refeeding on the abundance of FAS mRNA in liver of starved ducklings. At the beginning of the experiment the ducklings were 2 days old and unfed. Total RNA was extracted, fixed to GeneScreen, and hybridized to a single-stranded ^{32}P-labeled FAS cDNA. Five amounts of total RNA from goose liver (0.2—10 μg) were dotted onto each sheet of GeneScreen. The hybridization signals from the goose RNA samples were used to construct a standard curve and to normalize the results from different hybridizations. The concentration of FAS mRNA in the unknown samples is expressed as units/μg of total RNA, where one unit is the hybridization signal equivalent to that observed with 1 μg of the goose RNA. Each point is the mean ± SE of 3 experiments (1 duckling/experiment). There were essentially no changes in FAS mRNA concentrations when starved birds continued to starve. Taken from Back *et al.* (1986) with permission of the *J. Biol. Chem.* and to which the reader should refer for additional information.

fold. After a slight decrease at 12 h, the level was maintained at about 10 times that in starved ducklings. The half-life of FAS in fed ducklings, estimated from the time required to reach half its new steady state (Berlin and Schimke, 1965), was about 4 h. An independent but identical experiment, yielded a half-life of 6 h. In the reciprocal experiment, starvation of previously fed ducklings resulted in a prompt decrease in the abundance of FAS mRNA (Fig. 3) which reached a new steady state of about 10% the level in fed ducklings at 24 h after initiating starvation. We estimated a half-life of 3 h for FAS in the liver of starved ducklings (Fig. 3, inset). Feeding and starvation regulated both the large and the small FAS mRNAs in parallel (Back *et al.,* 1986). The small difference in rate of degradation of FAS mRNA between fed and starved ducklings could not account for the 10-fold difference in mRNA abundance between these 2 conditions.

If degradation is not the regulated step, then the production rate must be regulated. The rate of production of cytoplasmic FAS mRNA is a function of the rate of transcription of this gene, the rate of processing of the primary transcript and the rate of transport of the mature transcript from the nucleus to the cytoplasm. The rate of degradation of the nuclear precursor transcripts also could affect the rate of production of the mature mRNA. We determined the effects of starvation and refeeding on transcription of the FAS gene by measuring elongation of pre-existing transcripts in isolated nuclei using the transcription 'run-on' assay (Mory and Gefter, 1978; McKnight and Palmiter, 1979). RNA polymerase molecules engaged in transcription when nuclei are prepared remain bound to the DNA in a transcription complex. Under the *in vitro* conditions used in this assay, each polymerase elongates by a roughly constant amount, irrespective of the

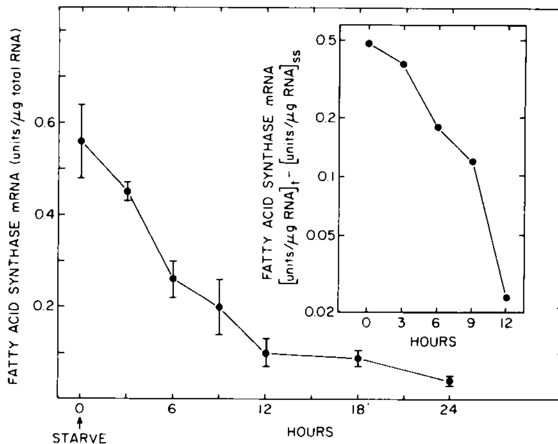

Fig. 3. The effect of starvation on the abundance of FAS mRNA in duckling liver. These birds were 11 days old and had been fed 8 days, starved 2 days, and refed 1 day prior to removing the food. This dietary regime ensured a high level of FAS mRNA at the beginning of the starvation experiment. Other conditions and procedures were as described in the legend to Fig. 2. The inset is a semi-log plot of the approach of FAS mRNA to the steady state ($[\text{units}/\mu\text{g RNA}]_{ss}$, the concentration of FAS mRNA in the starved state; $[\text{units}/\mu\text{g RNA}]_t$, the concentration at any time, *t*, during approach to steady state). There were essentially no changes in FAS mRNA concentration when fed birds continued to feed. Taken from Back *et al.* (1986) with permission of the *J. Biol. Chem.* and to which the reader should refer for additional information.

identity of the gene or the physiological condition of the cell or animal; initiation or reinitiation does not occur. Incorporation of labeled nucleotide triphosphate into RNA *in vitro* is thus a measure of the number of polymerase molecules engaged in transcription at the time the nuclei were prepared. The number of polymerase molecules engaged in transcription along a particular segment of DNA should be proportional to the rate of the pace-setting reaction. Initiation is usually the pace-setting step for transcription, but transcription elongation or premature termination also could be pace setting.

Transcription of the FAS gene was increased more than 8-fold within 45 min of refeeding. There was little change in the transcription rate over the next 5 h, or even after 24 h of feeding (Fig. 4). Between 24 and 30 h of refeeding there appeared to be a further increase in the transcription rate (Fig. 4, right panel). However, variability and the small number of observations at these time points make the apparent increases statistically insignificant. Transcription of the albumin gene was altered only slightly during these nutritional challenges and always in a direction opposite to that of the FAS gene (Goldman *et al.,* 1985). Refeeding starved ducklings did stimulate the transcription of total RNA but to a much smaller extent than the increase in transcription of the FAS gene or the comparable increase in concentration in FAS mRNA. Furthermore, transcription of the FAS gene was expressed relative to that of total RNA, thus factoring out changes in total transcription (Back *et al.,* 1986). In sum, these results indicate that most, if not all, of the increase in abundance of FAS mRNA which

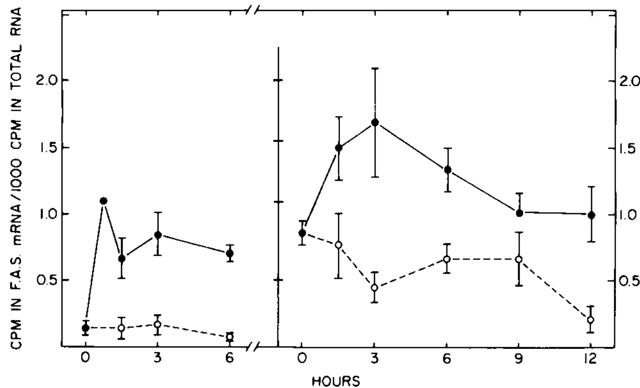

Fig. 4. The effects of feeding and starvation on transcription of the FAS gene. Ducklings were fed *ad libitum* for 10—14 days and starved for 48 h before beginning these experiments. Left panel : ducklings were fed (closed circles) or starved (open circles) as indicated; right panel : ducklings were fed for 24 h (starting with time zero of the left panel) and then starved (open circles) or fed (closed circles) as indicated. Nuclei isolated from the treated ducklings were used for the measurement of relative rate of transcription of the FAS gene as described in Back *et al.* (1986). The transcription rate for FAS (expressed as parts per thousand) = [cpm (pDFAS1—pBR322/cpm x 10^3 in total RNA] x 100/hybridization efficiency x 11 500 /1 350, where 11 500 is the average length of the 2 FAS mRNAs in nucleotides and 1 350 is the length of pDFAS1 in base pairs. The hybridization efficiency averaged about 50%. Hybridization to the paper containing pBR322 averaged about 100 cpm (0.005 parts per 1000). The results are expressed as means ± SE of 3—7 experiments; the lack of an error bar indicates that it was smaller than the symbol. In each experiment, nuclei were isolated from a different animal.

accompanies refeeding of a starved animal is due to increased transcription. Since 3'-fragments of the fatty acid synthase cDNA were used as probes in these estimates of transcription rates, we can not distinguish between regulation at initiation, at elongation or by premature termination.

Transcription is unlikely to be the only step involved in the regulation of FAS mRNA concentration, however, because transcription of the FAS gene decreased slowly when fed ducklings were starved (Fig. 4, right panel). Nine hours after food was removed, the rate of transcription of the FAS gene was still more than 50% of that in fed birds. At the same point in time, the level of FAS mRNA was only 35% of that in fed birds (Fig. 3). If transcription is the only regulated step, then the decay in mRNA concentration should lag behind the decay in transcription. Thus, posttranscriptional mechanisms may play a role in the decrease in FAS mRNA cause by starvation. These posttranscriptional mechanisms must be transient, however, because 48 h of starvation decreases both transcription and mRNA level by about 90%.

Mechanisms which regulate abundance of FAS in hepatocytes in culture

In hepatocytes prepared from the livers of 18—19-day-old chick embryos and incubated in a chemically-defined serum-free medium, FAS activity increased slowly without adding hormones. This increase in enzyme activity was comparable in amount and timing to the prenatal increase in FAS which precedes hatching. Both the *in vivo* and *in vitro* phenomena may reflect the removal of an inhibitor which normally represses expression of FAS in embryos which are less than 19 days of incubation. The addition of insulin alone or thyroid hormone alone caused 2-fold increases in FAS activity over the course of a 3 day incubation (Fischer and Goodridge, 1978). When insulin plus triiodothyronine were added to the medium, FAS activity increased more than 15-fold over that in cells without added hormone. Interestingly, insulin-like growth factor 1 (IGF-1) could substitute for insulin (Goodridge *et al.*, 1989a). IGF-1 was more effective than insulin at amplifying the induction of FAS by triiodothyronine and was maximally effective at much lower concentrations. The physiological significance of these effects of IGF-1 is unknown.

When glucagon was added with insulin and triiodothyronine (T_3), FAS activity in hepatocytes incubated for 3 days was 70—80% lower than that with insulin plus triiodothyronine alone (Fischer and Goodridge, 1978; Wilson *et al.*, 1986). The effects of glucagon were mimicked by the addition of cyclic AMP (Goodridge *et al.*, 1989b), consistent with the well-known mediation of the action of glucagon by this cyclic nucleotide. The relative rate of synthesis of FAS correlated positively with enzyme activity indicating that synthesis of enzyme was the hormonally regulated step (Fischer and Goodridge, 1978).

Except when T_3 alone was added, the abundance of FAS mRNA paralleled the rate of enzyme synthesis, indicating that regulation is primarily pretranslational (Wilson *et al.*, 1986). The addition of trriodothyronine alone had a much smaller effect on enzyme activity and enzyme synthesis than on mRNA concentration (Fischer and Goodridge, 1978; Back *et al.*, 1986). This observation suggests that insulin, in the presence of triiodothyronine, stimulated translation of the FAS mRNA.

Transcription of the FAS gene was measured in nuclei from hepatocytes incubated with insulin alone, insulin plus triiodothyronine and insulin plus triiodothyronine plus cyclic AMP. The run-on assay and 3'-cDNA probes were used for these experiments. Triiodothyronine stimulated transcription of the FAS gene (Fig. 5), whereas cyclic AMP inhibited gene transcription to about the same extent as it inhibited mRNA level. The relative effects of the hormones were the same for both of the FAS cDNAs which we used. Neither hormone affected transcription of the β-actin gene, indicating that the effects were selective for the FAS gene. We conclude that thyroid hormone and cyclic AMP regulate FAS activity by controlling the rate of transcription of its gene.

Components of the intracellular signalling pathway

When triiodothyronine was added to hepatocytes pre-incubated for 2 days with insulin, FAS mRNA accumulated with sigmoidal kinetics, approaching a new steady state about 48 h after the addition of triiodothyronine. Puromycin, an inhibitor of protein synthesis, blocked the effect of triiodothyronine (Wilson *et al.*, 1986). The same result was obtained with malic enzyme (Back *et al.*, 1986) another triiodothyronine-inducible lipogenic enzyme. The slow sigmoidal accumulation kinetics and dependence upon on-going protein synthesis suggests involvement of a protein intermediate in the action of triiodothyronine on expression of the FAS and malic enzyme genes.

Triiodothyronine is reported to stimulate protein kinase activity or phosphorylation of nuclear proteins (Nikodem *et al.*, 1977; Ruel *et al.*, 1986). The functional consequences of these actions of triiodothyronine are unknown. Nevertheless, the reported effects on protein kinase activity suggested the

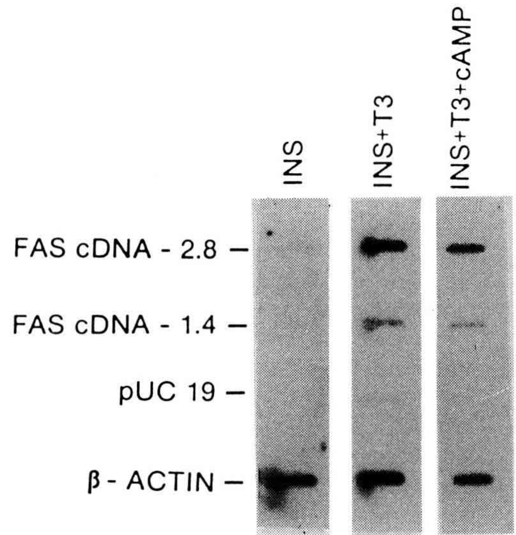

Fig. 5. The effects of triiodothyronine and cyclic AMP on transcription of the FAS gene in hepatocytes in maintenance culture. Hepatocytes were prepared from the livers of 18-day-old chicken embryos and incubated in a serum-free chemically-defined medium (Waymouth MD705/1) containing insulin (300 ng/ml) (Wilson *et al.*, 1986). The medium was changed to one of the same composition at about 20 h of incubation. At about 44 h of incubation, triiodothyronine (1 μg/ml) or nothing was added to the medium as indicated in the figure; nuclei were isolated from cells harvested at 68 h of incubation. Dibutyryl cyclic AMP (50 μM) was added to cells incubated with triiodothyronine for 24 h and the nuclei isolated after 6 h with dibutyryl cyclic AMP. Nuclei were prepared and the transcription run-on assay carried out essentially as described (Goodridge *et al.*, 1989b). Hybridizations and washes were carried out essentially as described by Linial *et al.* (1985). Chicken FAS cDNAs were kindly provided by Gordon G. Hammes (Yuan *et al.*, 1988). Vector DNA was used as a negative control. Actin DNA in pBR322 was used as a control for selectivity. Each slot contained 2 μg of DNA.

possibility that the putative protein intermediate might be a protein kinase or a protein kinase substrate. H-7, H-8 and HA1004, isoquinoline sulfonamide derivatives and non-specific inhibitors of protein kinase activity (Hidaka *et al.*, 1984), blocked the increases in FAS and malic enzyme mRNAs caused by triiodothyronine (Swierczynski *et al.*, submitted for publication). The effects of the protein kinase inhibitors on induction of malic enzyme and FAS mRNAs were evident 6 h after adding triiodothyronine. By contrast, total protein synthesis and some non-lipogenic enzymes were unaffected after 48 h of incubation with these inhibitors. Thus, the inhibitory effects on induction of the lipogenic enzymes were not due to general toxic effects of these drugs. We suggest that the abundances of FAS and malic enzyme mRNAs are regulated by a triiodothyronine-induced peptide intermediate which has a relatively long half-life. This intermediate may be a protein kinase or a substrate for a triiodothyronine-activated protein kinase.

Regulation of FAS in the uropygial gland

The uropygial gland of birds is a specialized sebaceous gland that produces ester waxes (Lucas and Stettenheim, 1972; Jacob and Ziswiller, 1982). Secretion is of the holocrine type and terminally differentiated cells filled with ester wax are continually being lost. Cell division in a population of basal cells furnishes new cells to replace those lost during secretion (Jenik *et al.*, 1987). Basal cells lie on a basement membrane which surrounds the tubules of the gland; they lack the large lipid droplets characteristic of mature sebocytes. Cells which push off the basal membrane rapidly differentiate into lipid-engorged sebocytes and are ultimately sloughed into the lumen of the tubule.

FAS accounts for 10% of the mass of soluble protein in the uropygial gland of young ducklings (Goodridge *et al.*, 1984). The basal cells, however, lack FAS (Jenik *et al.*, 1987). As the differentiating cells migrate from the basal membrane to the lumen of the tubule, they accumulate FAS (Jenik *et al.*, 1987). Based on preliminary *in situ* hybridizations, the accumulation of FAS during differentiation is accompanied by a similar accumulation of FAS mRNA (data not illustrated). The mechanism which triggers increased accumulation of FAS in the uropygial gland is unknown, but may involve growth factors secreted by the basal cells themselves or by mesenchymal cells which lie between the tubules.

Levels of FAS, rates of its synthesis and levels of its mRNA were measured in the uropygial glands of ducklings before and after hatching. All 3 processes were regulated in parallel. They were very low 7 days before hatching, began to increase about 4 days before hatching and approached the adult steady-state levels by the day of hatching. FAS and its mRNA increased more than 100-fold relative to the levels at 7 days before hatching (Goodridge *et al.*, 1984). The appearance of FAS and its mRNA in the embryonic uropygial gland preceded the prenatal increase in the levels of these macromolecules in liver. The onset of accumulation of FAS and its mRNA in the embryonic gland corresponded to the initial morphological appearance of mature sebocytes in the embryonic gland (Gomot, 1959) and probably represents the initial wave of differentiation in the gland.

While starvation dramatically decreased levels of FAS and its mRNA in

the livers of 1-day-old and 2-week-old ducklings, it had no effect on the abundance of these macromolecules in the uropygial gland (Goodridge *et al.*, 1984). We conclude : 1) that the signal(s) generated by starvation and which regulated hepatic FAS had no effect on the level of the enzyme or its mRNA in the uropygial gland; and 2) that, as in liver, regulation of FAS in the uropygial gland was exerted at a pretranslational step.

Structural characterization of the FAS mRNA and gene

The fatty acid synthases of liver and uropygial gland produce different long-chain fatty acids (Buckner *et al.*, 1978). Based on kinetic and immunologic properties of enzymes purified from both sources, others have concluded that the liver and uropygial gland enzymes are identical (Buckner *et al.*, 1978). Nevertheless, it remained possible that the enzyme in liver was produced from a different gene than the enzyme from the uropygial gland and that transcription from two different genes caused the different patterns of regulation in the two tissues. Experimental determination of the number of copies of the FAS in goose DNA indicated that there is only one gene per haploid genome (Back *et al.*, 1986). Further evidence for the unique nature of the FAS gene came from Southern blot analyses with 3 different cDNA probes (M13GFAS3A, M13GFAS3B, M13GFAS8) (Fig. 6). Each probe detected single fragments of identical sizes in genomic DNA from both liver and uropygial gland (Fig. 7). These results indicated that the same unique gene was transcribed into FAS mRNA in both tissues.

Southern analysis of liver and uropygial gland DNA with a fourth fragment of the FAS cDNA (M13GFAS3C) detected about 20 fragments in both tissues (Fig. 7, panel C). FAS3C lies between FAS3B and FAS8 (Fig. 6), both of which hybridized to fragments of the same size in genomic DNA digested with *Eco*RI, *Hind*III and *Pst* I. If the Southern blots probed with FAS3C were washed at high stringency, all of the signals except that which corresponded to the fragments detected by FAS3B and FAS8 were differentially eliminated. This suggested that FAS3C contained a sequence which was repeated a small number of times in the goose genome. Since FAS is regulated coordinately with several lipogenic enzymes, it seemed possible that these repeated elements might represent an identifier sequence for the lipogenic enzymes. The nucleotide sequence of FAS3 and much of FAS8 was determined. There were no long open

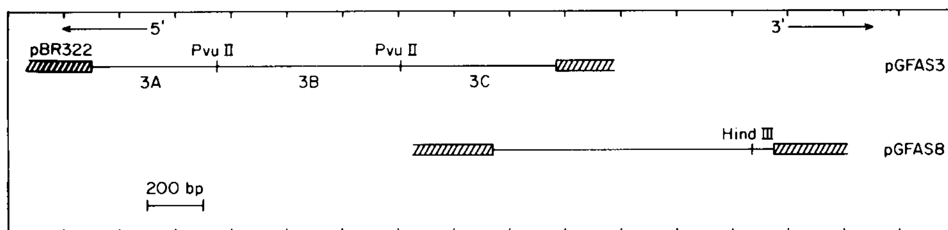

Fig. 6. Partial restriction map of cloned FAS cDNAs used in these experiments. The solid lines represent goose DNA. The cross-hatched bars represent pBR322 DNA. Taken from Back *et al.* (1986) with permission of the *J. Biol. Chem.*

reading frames, indicating that FAS3 and FAS8 corresponded to the 3'-non-coding region of FAS mRNA. The longer of the 2 FAS mRNAs thus contains a 3'-non-translated sequence of at least 2 600 nucleotides. The part of the cDNA corresponding to FAS3C contained an unusual structure — a polypyrimidine tract of 124 bases, consisting of 15.5 repeats of the octamer CCTTTCTT.

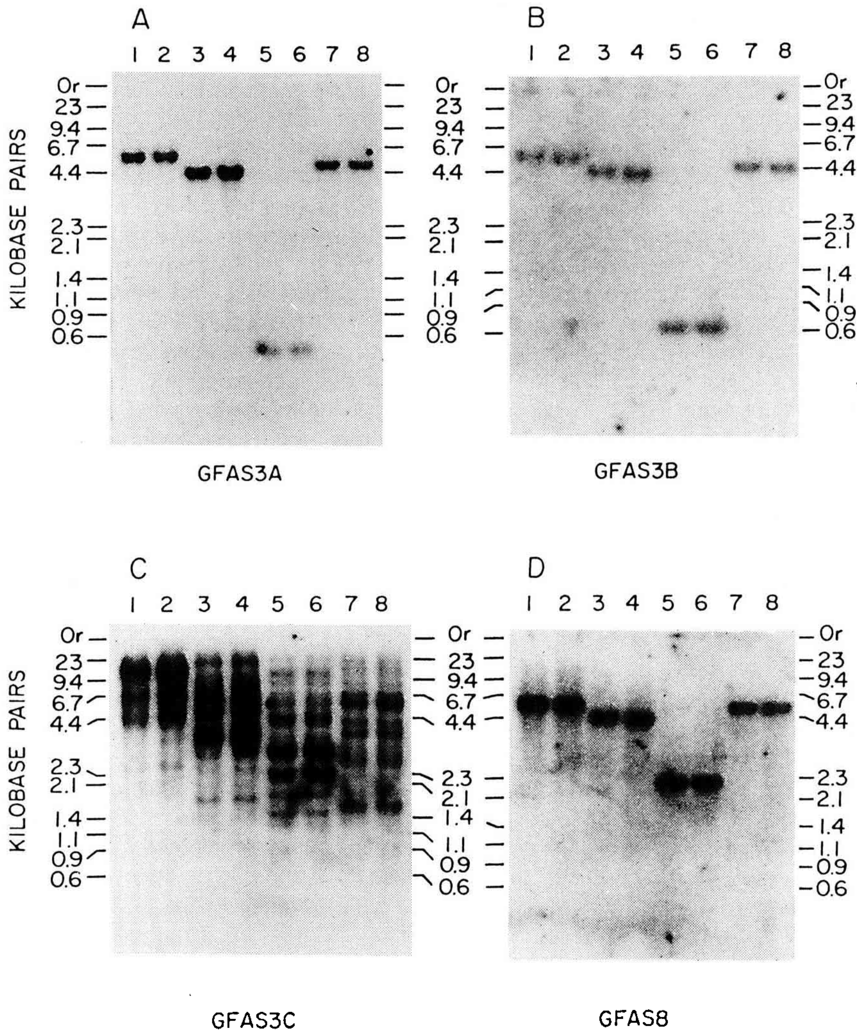

Fig. 7. Southern blot analysis of DNA from goose liver and uropygial gland using different cDNAs from the 3'-end of FAS cDNA as probes. Genomic DNA was isolated from goose liver and uropygial gland, digested with *Eco*RI (1, 2), *Hind*III (3, 4), *Pvu*II (5, 6), and *Pst*I (7, 8), separated by size on agarose gels, blot-transferred onto nitrocellulose membranes, and hybridized to ^{32}P-labeled, single-stranded cDNA probes (Back *et al.*, 1986). The probes were : A, pGFAS3A; B, pGFAS3B; C, pGFAS3C; D, pGFAS8. Note that pGFAS3B is flanked by *Pvu*II sites.

Polypyrimidine tracts are common in the genomic DNA of vertebrates (Deugau *et al.*, 1983), but rarely expressed as part of a messenger RNA. The polypyrimidine tract was probably responsible for the multiple restriction fragments detected by FAS3C in the Southern analyses.

Recombination can occur in plasmids during growth of the host bacteria, suggesting that the polypyrimidine tract might have been an artifact of cDNA cloning. The finding of one clone which had 16.5 repeats of the polypyrimidine tract made this possibility even more likely. A 40mer, $GGAAAGAA_5$, was synthesized on an Applied Biosciences oligonucleotide synthesizer. In a Northern blot analysis, the end-labeled 40mer hybridized to both FAS mRNAs in poly (A^+) RNA from both liver and uropygial gland of the goose (Fig. 8). Even though poly (A^+) RNA from duck liver contained intact FAS mRNAs of both sizes, it did not contain sequences which hybridized to the 40mer (Fig. 8). Since regulation of the levels of FAS in liver and uropygial gland of ducks is the same as that in geese, expression of the polypyrimidine tract in FAS mRNA is not a requirement for regulation. It remains possible, however, that the polypyrimidine tract is present in a non-mRNA region of the FAS gene of duck and plays a role in regulation of transcription.

Polypyrimidine tracts endow DNA with unusual properties (Wells, 1988) and are hypothesized to play roles in the regulation of some genes (McKeon *et al.*, 1984; Christophe *et al.*, 1985). Polypyrimidine tracts in super-coiled plasmid DNA induce sensitivity to single-strand-specific nucleases (Shen, 1983; Finer *et al.*, 1984) in or near the tracts. DNase hypersensitivity and S_1 sensitivity are also reported for regions of genes important in the regulation of transcription (Keene *et al.*, 1981; Weintraub, 1983). Super-coiled GFAS3 DNA in pBR322 contained two S_1-sensitive sites not present in pBR322 or pGFAS8 (data not shown). Genomic DNA from goose liver, however, did not appear to contain a DNase-sensitive site near the polypyrimidine tract (data not shown).

Modification of DNA by methylation of cytosines is another potential regulatory mechanism, especially with respect to tissue-specificity. We compared the methylation patterns of CCGG sequences in the 3'-region of the FAS gene. Methylation of the internal cytosine of this sequence was assessed with *Hpa*II and *Msp*I, a methylation-dependent and independent isoschizomer, respectively. FAS was hypermethylated on one of the cytosines in the cleavage site of the fragment generated by digestion with *Msp*I. The hypermethylated fragment was detected by hybridization with FAS3B (Fig. 9A) and was similar in size and intensity in DNA from both liver and uropygial gland. Tissue-specific expression of the FAS gene in liver and uropygial gland is thus unlikely to involve this hypermethylated site. Hypermethylation of the very actively transcribed FAS gene was unexpected because hypomethylation is usually associated with active genes (Cooper *et al.*, 1983 and references therein). The gene for malic enzyme, another lipogenic enzyme active in the uropygial gland and in the liver of fed animals, was hypomethylated on sites near its 3'-end (Fig. 9, panel C).

The adjoining cDNA fragment, FAS3C, detected about 20 bands when *Msp*I was used to cleave either liver or uropygial gland DNA. This was consistent with the multiple bands detected by this fragment in DNA digested with other restriction enzymes (Fig. 8). When *Hpa*II was used, virtually all 20 bands disappeared and were replaced by a broad band of about 23 kb (Fig. 9B). Thus, like the FAS fragment, each of the 20 or so genomic

fragments containing a polypyrimidine tract similar in sequence to $CCTTTCTT_{15}$ was flanked by one or more hypermethylated CCGG sequences. Again, the patterns were the same for both liver and uropygial gland, indicating that neither the polypyrimidine tract nor the hypermethylation sites flanking the tract were

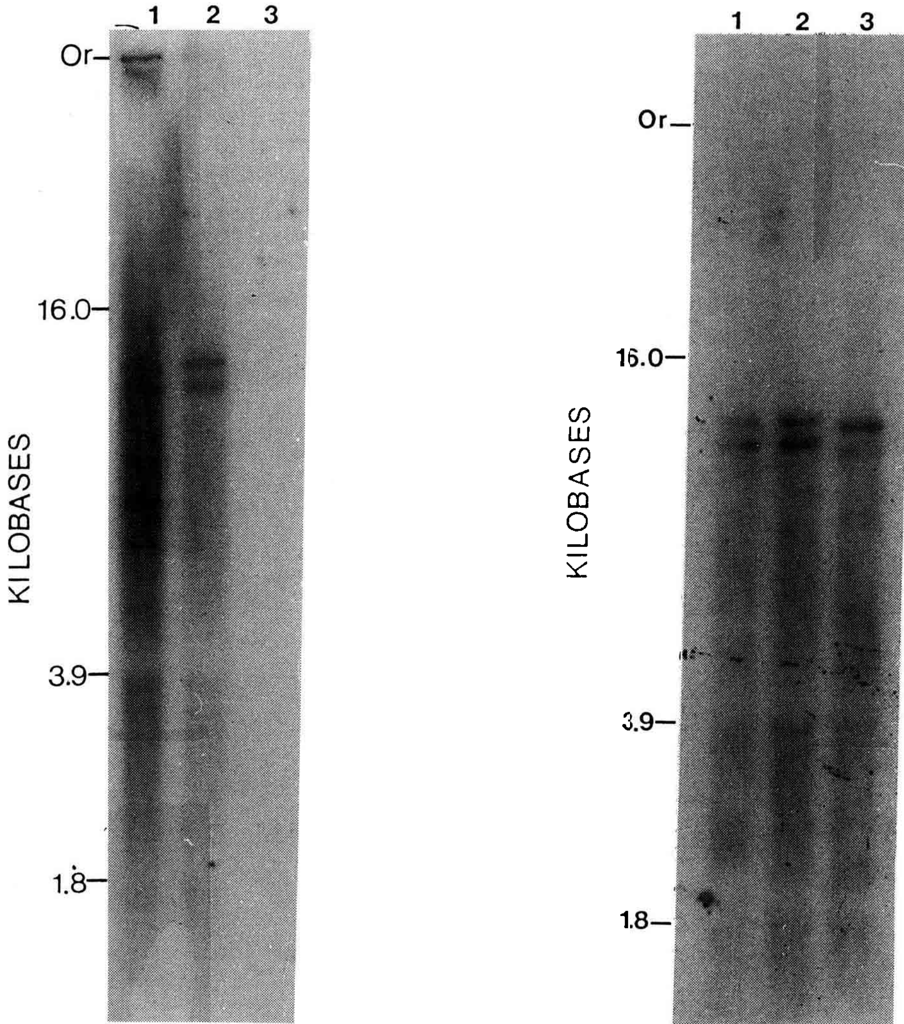

Fig. 8. The long and short FAS mRNAs from goose liver and goose uropygial gland contain the polypyrimidine tract; neither FAS mRNA from duck liver contains this sequence. Poly (A^+) RNA (15 μg) was subjected to electrophoresis in a 0.8% agarose gel buffered with morpholinepropanesulfonic acid (Back *et al.*, 1986) blot-transferred onto nitrocellulose membrane and hybridized to a synthetic ^{32}P-labeled polypyrimidine oligonucleotide (40mer) under aqueous conditions (left panel). The blot was washed to remove the probe and rehybridized with ^{32}P-labeled, single-stranded cDNA generated from M13GFAS3B (right panel). Sources of the RNAs were : lane 1, goose uropygial gland; lane 2, goose liver; lane 3, duckling liver.

involved in tissue-specific expression of the FAS gene. On the other hand, the finding that all of the fragments which contained this or a very similar poly-pyrimidine sequence were flanked by hypermethylated sites suggests a relationship between the presence of the polypyrimidine tract and methylation. The significance of the hypermethylation or of this putative relationship is unknown.

Isolation of cDNAs and genomic DNA for FAS

Understanding the transcriptional mechanisms which regulate FAS expression during differentiation and upon challenge by hormones or changes in diet will require the isolation and characterization of the gene for FAS, especially its 5'-flanking region. Our initial cDNA clones contained sequence complementary to about the 2 600 nucleotides at the 3'-end of FAS mRNA. Preparation of another cDNA library in a lambda vector using a combination of oligo (dT) and random primers for first-strand DNA synthesis has allowed us to isolate additional cDNAs from the more 5'-region of the mRNA. These cDNAs are being characterized.

We have also prepared genomic libraries of goose DNA in a cosmid vector

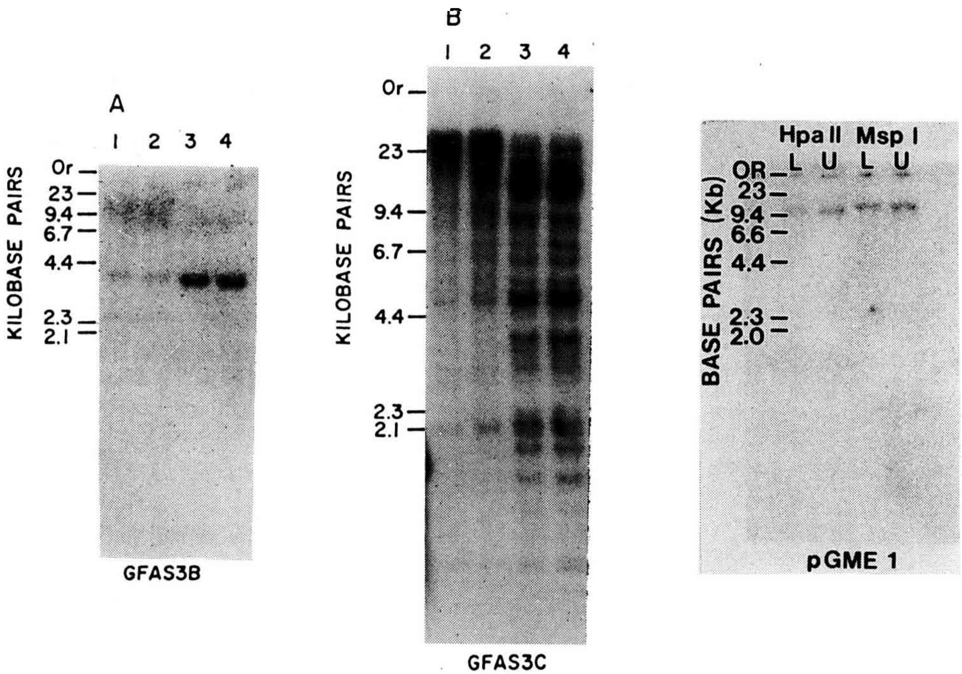

Fig. 9. Methylation at the 3'-ends of the FAS and malic enzyme genes and of DNA fragments containing a polypyrimidine tract similar in sequence to the one in the FAS mRNA. Genomic DNA was isolated from goose liver (lanes 1, 3 and L) and uropygial gland (lanes 2, 4 and U), digested with *Hpa*II (lanes 1, 2) and *Msp*I (lanes 3, 4), separated by size on agarose gels, blot-transferred onto nitrocellulose membranes and hybridized to ^{32}P-labeled, single-stranded cDNA probes. The probes were : A, pGFAS3B; B, pGFAS3C; C, pGME1.

(Ish-Horowicz and Burke, 1981). Five overlapping clones spanning more than 100 kb have been isolated and partially characterized (Fig. 10). Only the 3'-most of these clones (285-4 and 6-3-2) contain exon sequence corresponding to our original cDNA clones. Presence of exon in the other clones was verified by hybridization with end-labeled poly (A+) RNA extracted from the liver of a fed goose and by hybridization of labeled exon-containing genomic DNA fragments to FAS mRNA in Northern blot analyses. Some of our new cDNA clones have been isolated by screening the cDNA library mentioned above using labeled exon-containing genomic DNA fragments. At least one of the resulting cDNA clones extends substantially 5' of the existing genomic clones. It thus appears that the gene will be much larger than the approximately 90 kb of FAS genomic DNA which we have cloned so far. Our efforts to isolate cloned DNAs corresponding to the entire mRNA and the entire gene are continuing.

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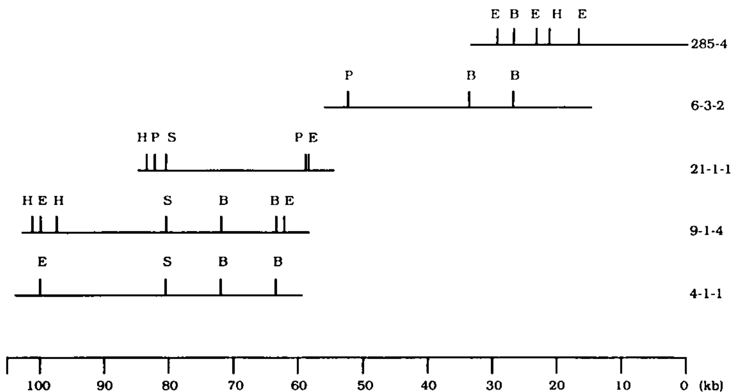

Fig. 10. Partial restriction maps of cloned FAS genomic DNA. Only the insert DNA is shown. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I; P, *Pst*I.

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