

Glucocorticoid Regulation of Adipocyte Differentiation: Hormonal Triggering of the Developmental Program and Induction of a Differentiation-dependent Gene

ALGER B. CHAPMAN, DAVID M. KNIGHT, and GORDON M. RINGOLD
Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT We have analyzed the hormonal basis for the acceleration of differentiation by dexamethasone and insulin in the stable adipogenic cell line TA1. These cells, which were derived from 5-azacytidine-treated 10T1/2 mouse embryo fibroblasts, undergo differentiation in culture after reaching confluence. The ensuing morphological changes are accompanied by widespread alterations in the pattern of protein synthesis and the increased accumulation of specific mRNAs. Using cDNA clones corresponding to mRNAs that are induced during adipogenesis, we find that dexamethasone elicits the precocious accumulation of differentiation-specific gene products. This effect appears to be mediated by the glucocorticoid receptor, yet unlike standard steroid inductions, most of the RNAs reach the same maximal levels in the absence of dexamethasone. Glucocorticoids thus may increase the expression of a regulatory factor required for activating the entire set of differentiation-dependent genes. We also describe a gene whose transcription is not only activated during adipogenesis but is also specifically inducible by dexamethasone in the mature adipocyte. Moreover, the glucocorticoid responsiveness of this gene in differentiated cells appears to be dependent on its prior developmental activation.

During development, cells that were pluripotent become increasingly restricted to specific differentiation pathways. This process, which culminates with differentiation into adult tissues, undoubtedly involves an ordered sequence of changes in gene expression reflected by synthesis of increasingly specialized proteins. Although little is known about how these changes are regulated, many of them involve interaction with specific inducers or hormones. Examples include induction of amphibian metamorphosis by thyroxine (15), stimulation of oviduct development by estrogen and progesterone (37, 41), and triggering of insect molting by ecdysone (49). Such hormonal effects may provide useful insights into the mechanisms underlying the developmental control of gene expression. Analysis of such mechanisms, however, has been hampered by the complexity of the systems being studied and the difficulty in obtaining pure populations of precursor cells that undergo differentiation under controlled conditions. To overcome this problem, we sought a cell line that can be induced to undergo differentiation in culture. We recently described the isolation and characterization of a stable adipogenic cell line, TA1, derived from 5-azacytidine-treated 10T1/2 mouse

embryo fibroblast (7). The cells are preadipocytes which during growth resemble the 10T1/2 fibroblasts. Once growth is arrested by allowing cells to reach confluence, they exhibit (over a period of 1–2 wk) the morphology characteristic of adipocytes and accumulate lipid droplets. This morphological change is accompanied by widespread alterations in the pattern of protein and RNA synthesis, and, as has also been found in other adipogenic cell lines (for review see reference 1), the appearance of enzymatic activities involved in fatty acid and triglyceride synthesis. We have isolated several cDNA clones from a lambda gt10 TA1 adipocyte library that correspond to mRNAs induced during adipogenesis (7) and have used these clones to begin characterizing the mechanisms controlling gene induction during the developmental conversion of these cells.

The differentiation of adipocytes in tissue culture is under the influence of many extracellular factors. In general, it is dependent on growth hormone and other unidentified serum factor(s) (28). Furthermore, the differentiation has been reported to be accelerated by many hormones and pharmacological agents including insulin (17), indomethacin (46), pros-

taglandin F2 (40), methyl isobutylxanthine (40), and a combination of dexamethasone and methylisobutylxanthine (39). The adipose conversion of TA1 cells is similarly accelerated by treatment with dexamethasone and insulin (7), resulting in lipid accumulation within several days and the precocious accumulation of specific RNAs for all of the adipose-induced cDNAs we have studied.

In this paper, we analyze the basis for the acceleration of TA1 adipocyte differentiation by dexamethasone and insulin and suggest that glucocorticoids control the expression of a regulatory factor required for triggering the expression of differentiation-dependent genes. We also examine the expression of a single gene whose mRNA is not only activated during adipocyte differentiation but is also specifically inducible by dexamethasone in the mature adipocyte. Moreover, the glucocorticoid responsiveness of this gene is dependent on its prior developmental activation.

MATERIALS AND METHODS

Cell Culture Conditions: 10T1/2 cells (32) and TA1 cells (7) were grown in Eagle's basal medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum. All serum used was heat inactivated at 56°C for 30 min. Cultures were grown in 100-mm petri dishes at 37°C in a humidified incubator at 5% CO₂ atmosphere. The medium was changed every 3 d.

Hormonal Treatment of Cells: To compare the acceleration of adipocyte differentiation by different hormonal treatments, TA1 cells were treated at confluence with either 1 μ M dexamethasone, 5 μ g/ml insulin, or a combination of both. Treatments were continued throughout the experiment. To generate a log dose response curve of the acceleration of clone 1 mRNA accumulation, TA1 cells were treated at confluence with varying concentrations of dexamethasone and harvested for RNA isolation after 3 d.

In analyzing the time course of clone 10 mRNA induction by dexamethasone, TA1 cells were maintained without treatment for 16 d after confluence to allow adipocyte differentiation. 1 μ M dexamethasone was then added and cells were harvested at varying times for RNA isolation. Similarly, to generate a log dose response curve of clone 10 mRNA induction, TA1 cells were allowed to differentiate 15 d without treatment, after which varying concentrations of dexamethasone were added. 3 d later, cells were harvested for RNA isolation.

To analyze clone 10 mRNA induction in preadipocytes and 10T1/2 cells, cells were seeded into 100-mm petri dishes such that confluence would not be reached after 3 d. These cells were treated with 1 μ M dexamethasone and harvested for RNA isolation 3 d later, while still subconfluent.

RNA Isolation: Total cell RNA was prepared by the method of Chirgwin et al. (8). Briefly, cells were suspended in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.2% *N*-lauroylsarcosine, 0.2 mM β -mercaptoethanol. The resulting cell lysate was layered on a 5.7 M cesium chloride cushion and centrifuged at 80,000 g for 19 h. The RNA pellet was collected, and its concentration was determined by reading absorbance at 260 nm. Poly(A)-containing RNA was isolated by oligo(dT)-cellulose (Bethesda Research Laboratories) chromatography, as described by Aviv and Leder (4).

Subcloning of Lambda gt10 cDNA Clones: Clone 1 and clone 10 were originally isolated from a lambda gt10 TA1 adipocyte cDNA library as described by Chapman et al. (7). The recombinants were digested with Eco R1 and ligated into the unique Eco R1 site of pEMBL 9 (11) using T4 DNA ligase. Recombinants were transfected into *E. coli* C600, and ampicillin-resistant transfectants were selected. Taking advantage of the location of the Eco R1 site in the β -galactosidase gene, recombinants were picked from Xgal indicator plates as galactosidase-negative colonies. Final verification of clone 1 and clone 10 cDNA subcloning was performed by hybridizing nick-translated parent lambda gt10 DNA to nitrocellulose blots containing Eco R1 cut recombinant plasmid. These subclones were then used for nick translation (see below).

Hybridization Conditions: Prehybridizations were performed overnight at 42°C with 50% formamide, 2 \times SSPE (1 \times SSPE consists of 0.18 M NaCl, 10 mM NaH₂PO₄, pH 7.7, 1 mM EDTA), 5 \times Denhardt's reagent (1 \times consists of 0.02% each of ficoll, polyvinyl pyrrolidone, and bovine serum albumin [BSA]), and 200 μ g/ml of denatured herring sperm DNA. Hybridizations were performed for 48 h under the same conditions with denatured ³²P-labeled nick-translated DNA probe (33). Filters were washed twice in 2 \times SSPE, 0.1% SDS at 50°C for 15 min followed by two washes in 0.2 \times SSPE, 0.1% SDS at 50°C for 20 min. They were then exposed to Kodak XAR-5 film at -70°C

with intensifying screens. To rehybridize filters with new probes, filters were washed in 10 mM Tris pH 7.4, 1 mM EDTA, at 70°C for 30 min, and then prehybridized as described above. Results were quantitated by densitometry using a GS3000 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Analysis of RNA: 10–15 μ g of total RNA or 10 μ g of poly(A)-containing RNA were treated with 2.2 M formaldehyde in 50% formamide, 10 mM NaH₂PO₄ pH 7.0, at 50°C for 15 min. Samples were then electrophoresed in a 1.4% agarose formaldehyde gel containing 2.2 M formaldehyde, 20 mM morpholine propane sulfonic acid pH 7.0, 5 mM sodium acetate, and 1 mM EDTA (25). The gels were briefly washed in water and incubated 30 min in 50 mM NaOH, 10 mM NaCl. Gels were then neutralized in 100 mM Tris pH 7.4 and incubated 1 h in 20 \times SSPE. RNA was then transferred to nitrocellulose using 20 \times SSPE as described by Thomas (43).

Nuclear Transcription Assays: Assays performed were a modification of the methods described by Vannice et al. (45). Nuclei were isolated from TA1 cells in the following manner: dishes containing cells were placed on ice, the medium was removed, and the cells were washed with 3 ml cold phosphate-buffered saline (PBS). 1 ml hypotonic buffer (20 mM Tris/HCl, pH 8.0, 4 mM MgCl₂, 6 mM CaCl₂, 0.5 mM dithiothreitol) was added and the dishes were held on ice for 5 min. Lysis buffer (0.6 M sucrose, 0.2% Nonidet P-40, 0.5 mM dithiothreitol) was added (1 ml), and the dishes were scraped with a rubber policeman. The cells were then homogenized in a tight-fitting Dounce homogenizer. Nuclei were pelleted by centrifugation at 500 g, washed once in resuspension buffer (0.25 M sucrose, 10 mM Tris/HCl pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol), repelleted at 500 g, and resuspended in reaction buffer (50 mM HEPES pH 8.0, 90 mM NH₄Cl, 5 mM MgCl₂, 0.5 mM MnCl₂, 2 mM dithiothreitol, 0.1 mM EDTA, 0.4 mM each of ATP, CTP, and GTP, 10% glycerol, 10 μ g/ml BSA) at a concentration of $\sim 10^8$ nuclei/ml, α -³²P-UTP (~ 600 Ci/mmol, ICN K & K Laboratories Inc., Plainview, NY) was added to a concentration of 2 mCi/ml, and the nuclei were incubated for 30 min at 26°C with occasional shaking. The tubes were placed on ice to stop the reactions.

RNA was prepared from the nuclei by a modification of the method of Smith et al. (42). The nuclei were pelleted and resuspended in 1 ml Tris/HCl pH 7.8, 0.3% SDS, 100 μ g/ml yeast RNA as carrier, and transferred to new tubes. The reaction tubes were rinsed with 1 ml 100 mM sodium acetate, 20 mM EDTA pH 5.0, and the solution was added to the nuclear lysate. The nuclear lysate was extracted with water-saturated phenol. After removal of the aqueous phase, the phenolic phase was reextracted with 50 mM sodium acetate, pH 5.0, 10 mM EDTA, 0.2% SDS. The aqueous phases were combined, neutralized with 1 M Tris/HCl pH 8.0, and the RNA was precipitated with 2.5 vol of ethanol. The pellets were resuspended in water and extracted once with phenol/chloroform (1:1), reprecipitated with ethanol, and resuspended in water.

Nitrocellulose filters (7-mm disks, Schleicher & Schuell, Inc., Keene, NH) containing cDNA were prepared by denaturing 3 μ g of linear plasmid DNA at 100°C in 0.1 N NaOH for 5 min, adding 1.5 vol of 20 \times SSPE, and applying the mixture to the filters (presoaked in 20 \times SSPE) using a dot-blot apparatus (Bethesda Research Laboratories). The filters were air dried and baked for 2 h in vacuo at 80°C. Filters were prehybridized for at least 4 h in 3 \times SSPE, 5 \times Denhardt's reagent (9), 200 μ g/ml yeast RNA, 1% SDS, 50% formamide. The ³²P reaction products ($\sim 10^7$ cpm) were hybridized to the cDNA-containing filters in 200 μ l of the same buffer used for prehybridization except that dextran sulfate was added to 4.8% (16). Hybridization was for 3–4 d at 42°C with shaking. After hybridization, the filters were washed twice at room temperature in 2 \times SSPE, 0.1% SDS for 15 min followed by two washes, 30 min each, in 0.1 \times SSPE, 0.1% SDS at 60°C. The filters were then exposed to x-ray film at -70°C with a Cronex intensifying screen.

RESULTS

Hormonal Control of Adipogenesis

As previously described, treatment of confluent TA1 cells with a combination of 1 μ M dexamethasone and 5 μ g/ml insulin accelerates their morphological adipose conversion and leads to precocious accumulation of adipose-inducible RNAs (7). This effect is shown in Fig. 1 for clone 1, a representative gene whose mRNA is regulated in an on/off fashion during adipose conversion. Nick-translated clone 1 DNA was hybridized to filters containing RNAs isolated from TA1 cells treated with various hormone regimens. To correct for differences in the amounts of RNAs loaded in each lane and in transfer efficiency, results were normalized to signals generated by probing with β -actin (gift from P. Gunning and

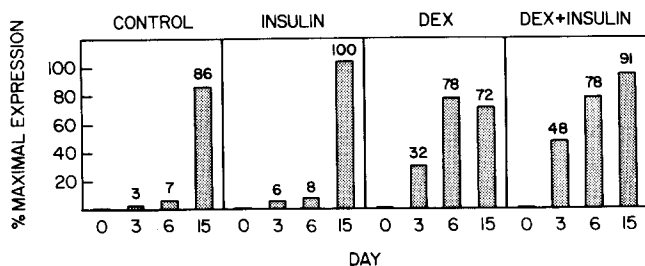


FIGURE 1 Hormonal effects on clone 1 RNA accumulation during adipocyte differentiation. The Northern blot described in Fig. 2 was scanned by densitometry. Results were normalized to β -actin signals (see text) and quantitated as a percentage of maximal clone 1 RNA accumulation in these samples. TA1 preadipocytes were treated at confluence (day 0) with either 1 μ M dexamethasone (DEX), 5 μ g/ml insulin, or a combination of both. Treatments were continued throughout the experiment.

L. Kedes). We have found that in TA1 cells, actin mRNA levels remain constant after confluence (Chapman, A. B., unpublished results).

Treatment with a combination of 1 μ M dexamethasone and 5 μ g/ml insulin resulted in 48% of maximal clone 1 expression after 3 d as compared with 3% in control cells. By 15 d of treatment, though, both control and treated cells have reached similar plateau levels of expression. To ascertain whether this hormonal acceleration of clone 1 mRNA induction was due to dexamethasone or insulin alone, or to a synergistic effect of the two, we compared the induction of clone 1 mRNA by each hormone alone. In a representative experiment, by 3 d after confluence with 1 μ M dexamethasone treatment, 32% of maximal clone 1 mRNA expression is reached as compared to 6% in 5 μ g/ml insulin-treated cells. By 6 d of treatment, 78% of maximal clone 1 expression is reached in dexamethasone-treated cells (as compared to dexamethasone- and insulin-treated cells), whereas only 8% of maximal expression is observed in insulin-treated cells and 7% in control cells. Though the time courses vary, similar results have been obtained for other clones we have studied (clones 1, 9, 11, 28, and 48). Thus, most if not all of the hormonal effect on precocious gene expression seems to be due to glucocorticoid treatment. The results of this experiment are qualitatively similar to those seen in two other independent experiments; since the timing of differentiation varies somewhat from experiment to experiment, it is not practical to present an average of these.

Radiolabeled glucocorticoid binding experiments have confirmed that both 10T1/2 and TA1 cells contain high affinity glucocorticoid receptors and that they are functional in stimulating transcription from the mouse mammary tumor virus promoter (Northrop, J., and Ringold, G., unpublished). To further characterize the nature of glucocorticoid action of TA1 adipocyte differentiation, we analyzed the dexamethasone dose dependence of clone 1 induction 3 d after treatment. A half maximal response is seen at \sim 10 nM dexamethasone (Fig. 2), a value consistent with that seen in other systems where the glucocorticoid response is known to be mediated through the glucocorticoid receptor (36, 44).

Clone 10, A Glucocorticoid-inducible Gene

When characterizing the hormonal acceleration of gene induction by dexamethasone and insulin, we found that clone 10 RNA, unlike the other RNAs studied, did not reach similar

plateau levels in treated and control cells. Fig. 3 shows an autoradiograph of a nitrocellulose blot containing RNA samples from TA1 cells treated with various hormone regimens that had been hybridized to nick-translated clone 1 and clone 10 DNA. Whereas clone 1 RNA reaches similar levels by 12 d after confluence in treated and control cells, clone 10 RNA reaches much higher levels (\sim 25-fold) in cells treated with dexamethasone or dexamethasone and insulin than that found in control or insulin-treated cells. A longer exposure of this autoradiograph (Fig. 3B) shows that clone 10 RNA is indeed activated during adipocyte differentiation in untreated cells, reaching a low plateau level by 12 d after confluence.

These results were analyzed by densitometry and quantitated as a percent of maximal clone 10 RNA expression (Fig. 4). TA1 cells treated with a combination of dexamethasone and insulin reached \sim 25-fold higher levels of clone 10 RNA than did control cells. Furthermore, this effect seems to be entirely due to dexamethasone, as insulin-treated cells show no greater expression than do control cells. In contrast, dexamethasone-treated cells reach a plateau level of clone 10 RNA that is indistinguishable from that of dexamethasone- and insulin-treated cells. The apparent decrease in clone 10 RNA seen at days 3 and 6 in dexamethasone- and insulin-treated cells relative to the dexamethasone-treated cells has not been seen reproducibly, however further investigation is warranted.

The behavior of the clone 10 gene is most easily explained if in addition to being activated during adipocyte differentiation, it is specifically responsive to glucocorticoid hormones. To test this hypothesis, fully differentiated adipocytes that

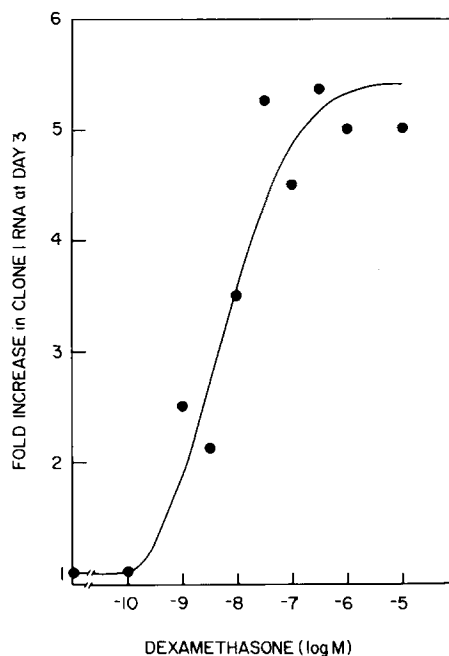


FIGURE 2 Dose dependence of the acceleration of clone 1 RNA induction by dexamethasone in differentiating adipocytes. TA1 cells were treated at confluence with varying concentrations of dexamethasone, and RNA was isolated after 3 d as described under Materials and Methods. 10 μ g of total RNA was electrophoresed in a formaldehyde gel and transferred to nitrocellulose filters. The filters were then hybridized with nick-translated clone 1 DNA, autoradiographed, and scanned by densitometry. Results are the average of two experiments normalized to β -actin signals (see text) and presented as the fold increase from untreated cells.

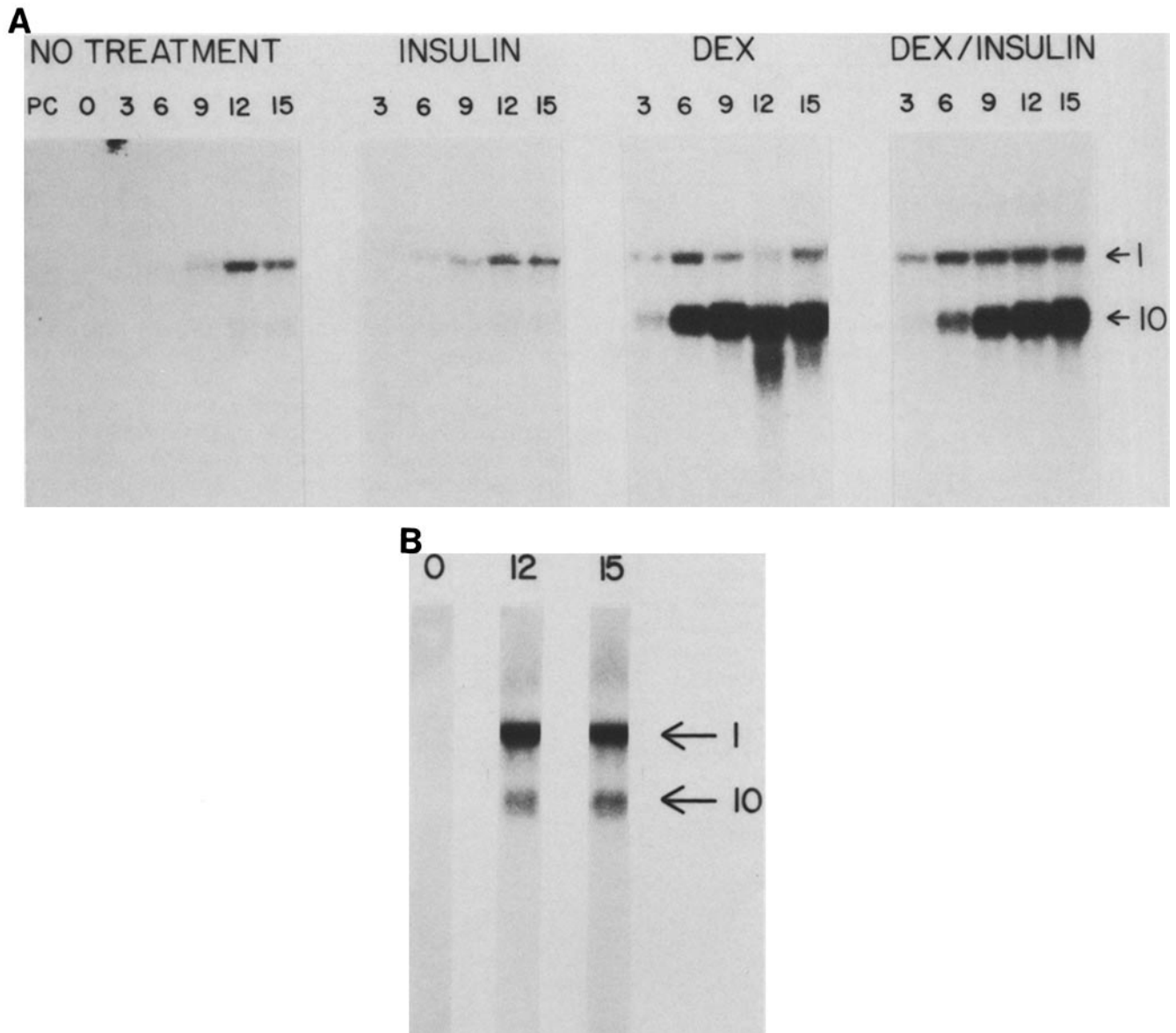


FIGURE 3 Time course of clone 1 and clone 10 RNA accumulation in control and hormone-treated cells. (A) TA1 preadipocytes were treated at confluence with either 1 μ M dexamethasone (DEX), 5 μ g/ml insulin, or a combination of both. RNA was isolated from cells 1 d before confluence (PC), at confluence (0), and every 3 d postconfluence with continual hormone treatment (3–15). 10 μ g of total RNA were electrophoresed in an agarose formaldehyde gel and transferred to a nitrocellulose filter as described in Materials and Methods. This blot was then hybridized to nick-translated clone 1 and clone 10 DNA. Clone 1 RNA is 4.3 kb, and clone 10 RNA is 2.3 kb. (B) Fourfold longer exposure of this filter showing lanes of RNA from cells receiving no hormones isolated at confluence, 12, and 15 d after confluence.

had never been exposed to hormone were treated with 1 μ M dexamethasone. In this experiment, dexamethasone treatment led to an approximately 2.5-fold induction of clone 10 RNA by 25 h of treatment, and a maximal 10-fold induction after 3 d (Fig. 5). The slow time course of this induction might be explained by a long half-life of clone 10 RNA, which would also account for our finding only a small decrease in clone 10 RNA 6 d after washing out the hormone (unpublished results). None of the other adipose-induced clones studied to date has shown this induction by dexamethasone once differentiation is complete. Thus, the action by dexamethasone in inducing clone 10 RNA seems to be separate and in addition to its effect on accelerating adipocyte differentiation.

We analyzed a dose response curve of clone 10 mRNA induction after a 3-d treatment with dexamethasone in differ-

entiated adipocytes. The half maximal response (shown in Fig. 6) occurs with \sim 10 nM dexamethasone, a value that as noted above is characteristic of glucocorticoid-receptor mediated responses. In addition, clone 10 RNA induction by 100 nM dexamethasone is significantly reduced by addition of 10 μ M progesterone, a competitive inhibitor of glucocorticoid binding to its receptor (Fig. 6, *inset*). Thus, the induction of clone 10 RNA appears to be mediated through the well characterized glucocorticoid receptor.

Differentiation Is Required for Clone 10 RNA Inducibility by Glucocorticoids

To test whether clone 10 RNA is glucocorticoid-inducible before its differentiation-dependent activation, 10T1/2 cells

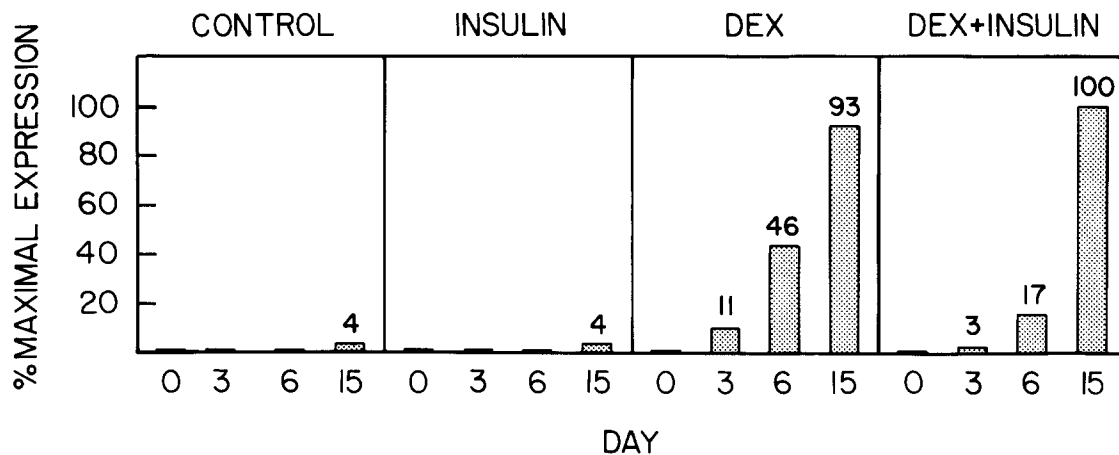


FIGURE 4 Hormonal effects on clone 10 RNA accumulation during adipocyte differentiation. The Northern blot described in Fig. 2 was scanned by densitometry. TA1 preadipocytes were treated at confluence (day 0) with either dexamethasone (DEX), insulin, or a combination of both. Results were normalized to β -actin signals (see text) and quantitated as a percentage of maximal clone 10 RNA expression in these samples.

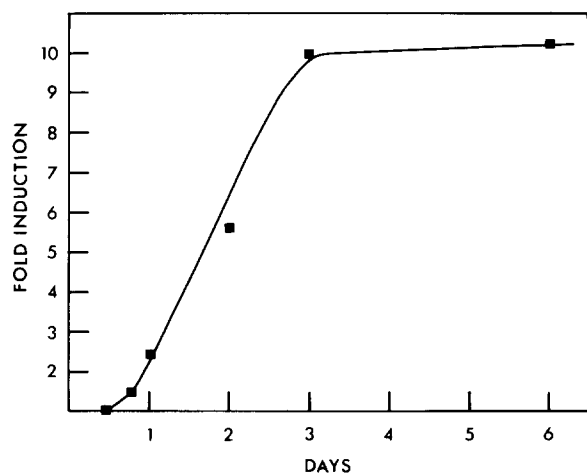


FIGURE 5 Time course of clone 10 RNA induction by dexamethasone in differentiated TA1 adipocyte. TA1 cells were allowed to differentiate 16 d without treatment (day 0). The mature adipocytes were then treated with $1 \mu\text{M}$ dexamethasone, and RNA was isolated at the times indicated. $10 \mu\text{g}$ of total RNA were run in an agarose formaldehyde gel, transferred to a nitrocellulose filter, and hybridized to nick-translated clone 10 DNA as described in the text. The results were quantitated by densitometry and plotted as the fold induction of clone 10 RNA for dexamethasone-treated cells compared with control cells. There was a small increase in the control clone 10 RNA levels during the course of this experiment.

and TA1 preadipocytes (preconfluent) were treated for 3 d with $1 \mu\text{M}$ dexamethasone. $10 \mu\text{g}$ of poly(A)-containing RNA from such cells (corresponding to $\sim 300 \mu\text{g}$ of total RNA) were fractionated on an agarose formaldehyde gel and transferred to nitrocellulose paper. For comparison, $10 \mu\text{g}$ of total RNA from TA1 adipocytes that were treated for 3 d with dexamethasone were also analyzed (Fig. 7). There is no detectable clone 10 mRNA in treated or control 10T1/2 cells despite the over 30-fold excess of poly(A)-containing RNA when compared with the glucocorticoid-treated mature adipocytes. Similarly, confluent 10T1/2 cells treated with the identical hormone regimens described in Fig. 2 for TA1 cells show no clone 10 RNA expression (unpublished results).

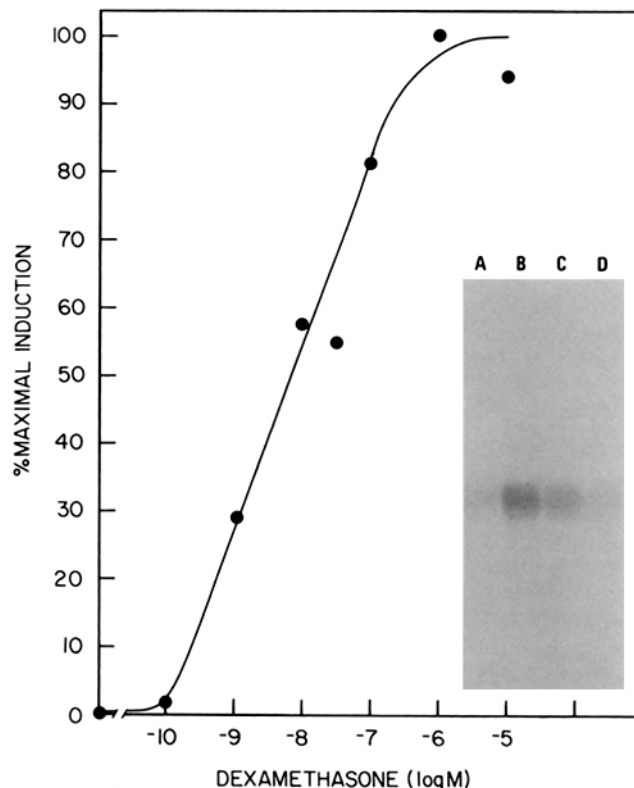


FIGURE 6 Dose dependence of clone 10 RNA induction by dexamethasone in mature TA1 adipocytes. TA1 cells were allowed to differentiate 15 d without treatment. They were then treated for 3 d with varying concentrations of dexamethasone. RNA was isolated, and $15 \mu\text{g}$ of total RNA were electrophoresed in an agarose formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with nick-translated clone 10 DNA as described in Materials and Methods. Results were quantitated by densitometry and are expressed as a percentage of maximal clone 10 RNA expression in this experiment. (Inset) Effect of progesterone on clone 10 RNA induction by dexamethasone. TA1 cells were differentiated in the absence of steroids for 15 d and then treated for 3 d with: (A) no addition; (B) 10^{-7} M dexamethasone; (C) 10^{-7} M dexamethasone + 10^{-5} M progesterone; (D) 10^{-5} M progesterone.

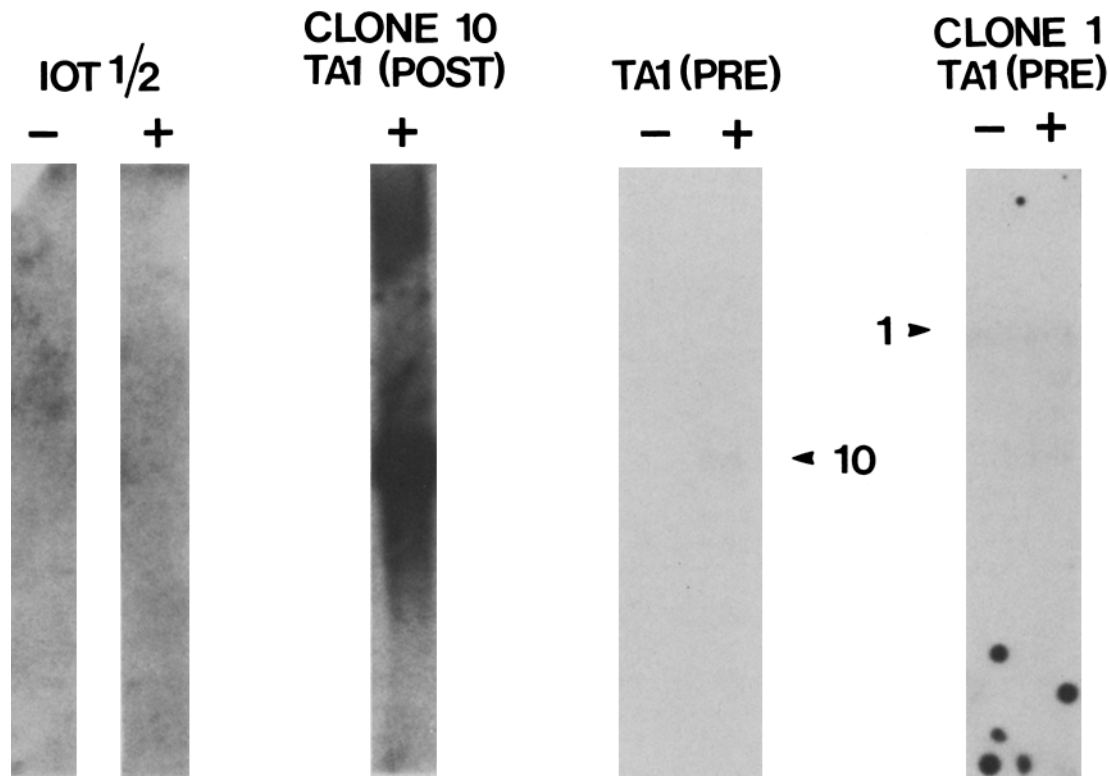


FIGURE 7 Analysis of clone 10 RNA expression and dexamethasone inducibility in 10T1/2 cells and TA1 preadipocytes. Preconfluent 10T1/2 cells and TA1 preadipocytes (PRE) were grown in the presence (+) or absence (-) of 1 μ M dexamethasone. 10 μ g of poly(A)-containing RNA was isolated from these subconfluent cells and electrophoresed in an agarose formaldehyde gel as described in the text. 10 μ g of total RNA isolated from mature TA1 adipocytes that had been treated for 3 d with dexamethasone (POST) was included for comparison. RNA was transferred to a nitrocellulose filter and probed with nick-translated clone 10 DNA. After exposure to film, the filter was washed free of probe and hybridized with nick-translated clone 1 DNA.

TA1 preadipocytes do show a small amount of clone 10 mRNA in the dexamethasone-treated cells. Calculations using results from densitometry scanning, which take into account the relative excess of poly(A)-containing RNA in these samples, show that the level of clone 10 RNA expression in these cells is \sim 9,000-fold lower than that seen in the mature TA1 adipocytes similarly treated with dexamethasone. Probing of this filter with nick-translated clone 1 DNA (Fig. 7) reveals a similar low level of clone 1 RNA expression in both treated and control preadipocytes, which could reflect either a very low level of expression in all preadipocytes or a small background of differentiating adipocytes in the preadipocyte population. We feel the latter is more likely because small areas of confluence can often be detected in subconfluent cultures, and TA1 differentiation is not synchronous. The low levels of clone 10 RNA, thus, more likely reflect dexamethasone-mediated induction in a small number of differentiated adipocytes rather than expression in preadipocytes.

Transcriptional Induction of Clone 10 mRNA Synthesis

Relative transcription rates of the clone 10 and clone 1 genes were measured using a nuclear transcription assay. TA1 cell nuclei were isolated from cells treated with 1 μ M dexamethasone and from untreated control cells and then incubated with α - 32 P-UTP at 25°C to allow extension and labeling of nascent RNAs. Under these conditions, transcriptional initiation does not occur (10, 27, 48); therefore incorporation

of UTP into newly synthesized RNA reflects the number of RNA polymerase molecules transcribing the gene of interest at the time the nuclei were isolated (18, 27). The labeled RNA was hybridized to filters containing immobilized clone 1 or clone 10 DNA. In control experiments, the amount of DNA used on the filters (3 μ g) was found to be in excess of that required to obtain maximal hybridization for clone 1 and clone 10 RNAs. Although no transcription of the clone 1 gene can be detected in preadipocytes (Fig. 8), by 6 d after confluence, transcription reached a plateau level reflecting its activation during adipocyte differentiation. In dexamethasone-treated cells, a similar plateau level of clone 1 transcription is reached. Although clone 10 gene transcription is also not detectable in adipocytes and reaches a plateau transcription rate in both treated and control cells, a significantly higher level is found in dexamethasone-treated cells. Thus, the specific induction of clone 10 RNA by dexamethasone is in large part, if not completely, transcriptional.

DISCUSSION

Glucocorticoid Acceleration of Adipocyte Differentiation

We have previously described the stimulation of TA1 adipocyte differentiation by treatment with dexamethasone and insulin (7). At 6 d after confluence, this effect can be seen both morphologically by the increased number of lipid-containing cells and biochemically by the more rapid induction

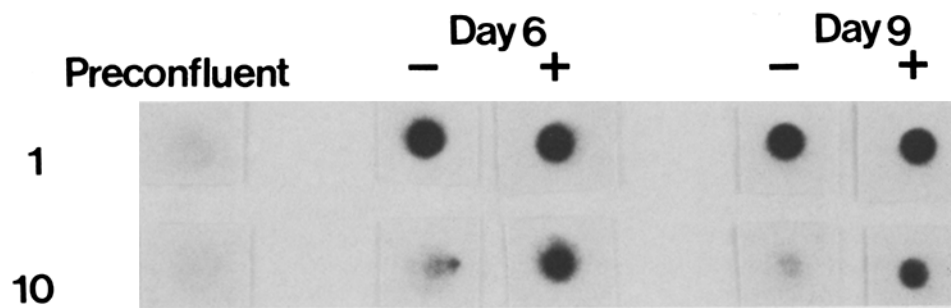


FIGURE 8 Transcriptional induction of clone 10 by dexamethasone. TA1 preadipocytes were grown after confluence in the presence (+) or absence (-) of 1 μ M dexamethasone. Nuclei were isolated at the indicated times and 32 P-UTP was incorporated into nascent RNA chains as described under Materials and Methods. Labeled RNA (10×10^6 cpm) was then hybridized for 4 d to 3 μ g of clone 1 or clone 10 DNA immobilized on nitrocellulose filters. After washing, filters were exposed to x-ray film for 3 d.

of adipose-specific RNAs. By 2 wk after confluence, though, an equal number of lipid-containing cells are found in control and treated cells. Similarly, as shown here for clone 1 RNA, the plateau level of most of these RNAs is similar in control and treated cells. Thus, hormone treatment seems to accelerate the normal differentiation process.

Results presented here demonstrate that dexamethasone is the hormone primarily responsible for this precocious induction of adipose RNAs. Several groups studying similar mouse adipogenic cell lines have reported acceleration of differentiation by insulin alone when analyzed morphologically (17), by lipid accumulation (17), and by induction of enzymatic activities (1, 47). We have not found a consistent effect in TA1 cells of insulin on specific RNA accumulation, despite the finding that these cells contain functional insulin receptors (unpublished observations). This discrepancy could be due to differences in cell lines or alternatively, as suggested by Rosen et al. (38), the effect of insulin might be predominantly mediated through its known role in stimulating enzymatic activities involved in lipid accumulation (9, 22). Thus, insulin would hasten the morphological appearance of lipid droplets without affecting the machinery that activates adipose genes.

The pattern of clone 1 gene expression during differentiation in the presence of dexamethasone is unlike that of a typical glucocorticoid-inducible gene because there is no difference in the maximal levels of RNA attained in treated and control cells. Rather, the effect of glucocorticoids seems to be on the mechanism that triggers the final differentiation event, thus accelerating the adipose conversion. Consistent with this notion, neither clone 1 RNA nor most of the other RNAs studied are inducible by dexamethasone in fully differentiated cells. An attractive hypothesis is that glucocorticoids may increase the production of a rate limiting factor(s) required for initiating adipocyte differentiation. Consistent with this notion we find that dexamethasone indeed accelerates the transcriptional activation of the adipose-specific genes (Knight et al., manuscript submitted for publication). Analysis of proteins or RNAs that are induced shortly after dexamethasone treatment may allow identification of such a factor.

A General Role for Glucocorticoids in Development

Glucocorticoids do not appear to be absolutely required for the triggering of adipocyte differentiation as untreated cells undergo adipocyte conversion albeit at a slower rate. We have

recently performed experiments using serum from which endogenous glucocorticoids have been removed by treatment with charcoal-dextran and find that the rate and extent of TA1 cell differentiation is unaltered. Thus, it is unlikely that low levels of hydrocortisone in serum simply parallel the dexamethasone effect. Rather, there may be two or more independent but functionally analogous pathways for triggering differentiation, one of which is glucocorticoid regulated. It is conceivable that glucocorticoids do not initiate the determining process itself, but rather may play a role in accelerating the maturation process once differentiation has begun. It is striking that during most of fetal development in mammals, cortisol levels remain low (29) despite the presence of glucocorticoid receptors in most tissues (5). Late in gestation there is a rise in circulating cortisol levels coincident with the maturation of many tissues (29). Glucocorticoids have been implicated in stimulating maturation of the small intestine and lung (3, 24), the induction of enzyme systems in various differentiating tissues (19, 26, 30), and acceleration of the appearance of acetylcholine receptor clusters during myotube development in culture (Blau, H., and I. Kaplan, personal communication). Thus, a major role of glucocorticoids in development may be in accelerating prenatal maturation and differentiation of tissues and enzyme systems that facilitate extrauterine survival (23).

Clone 10 RNA Is Induced by Glucocorticoids in TA1 Adipocytes

We have shown that unlike the other adipocyte-specific RNAs we have studied, clone 10 RNA is induced by glucocorticoids in fully differentiated adipocytes. This induction is most likely mediated by the glucocorticoid receptor as indicated by a characteristic log-dose response curve and antagonism of the induction by progesterone. Furthermore, the induction is at least in large part due to a stimulation of clone 10 transcription by treatment with dexamethasone.

Although these features are characteristic of the well studied primary transcriptional inductions by glucocorticoids of mouse mammary tumor virus (34, 35), metallothionein (20), and lysozyme (31) genes, we have not yet determined whether clone 10 mRNA induction is independent of protein synthesis (i.e., reflective of direct activation by the glucocorticoid-receptor complex). These experiments have been complicated by the slow time course of the induction, which might reflect either a long half-life for clone 10 RNA or the requirement

for induction and synthesis of a factor that mediates the hormonal effect on clone 10 gene expression. Binding studies with purified glucocorticoid-receptor complexes and genomic fragments of clone 10 may help in resolving this issue.

The induction of clone 10 RNA in mature TA1 adipocytes probably reflects a role for glucocorticoids in adipocytes distinct from that seen during adipocyte differentiation. Glucocorticoids are known to affect lipid metabolism as evidenced by the redistribution of adipose stores in the hypercorticoid state (for review see reference 12). They are also known to facilitate the lipolytic effects of various agents. For example, Fain et al. (13) showed that dexamethasone alone stimulates fatty acid release from adipose tissue. Addition of growth hormone, which alone has no effect, led to a dramatic rise in fatty acid release. This effect was found to be dependent on both protein and RNA synthesis. Interestingly, in another adipogenic cell (3T3-L1), glucocorticoids regulate adipocyte responsiveness to β -adrenergic agents by inducing a switch in receptor subtype expression from $\beta 1$ to $\beta 2$ (21). The basis for these actions of glucocorticoids are not well understood. The identification of the protein encoded by clone 10 RNA as well as the characterization of any other similarly induced genes in TA1 adipocytes may better define the role of glucocorticoid hormones in mature adipose tissue.

Acquisition of Glucocorticoid Responsiveness by Clone 10 RNA during Adipocyte Differentiation

During TA1 adipocyte differentiation, clone 10 mRNA is induced in the absence of hormone treatment, reaching a plateau level within 12 d after confluence. Its expression during differentiation in the presence of dexamethasone is thus affected by at least two factors; the hormonal induction of the general differentiation mechanism, and the specific hormonal induction of clone 10 RNA. The relative importance of each factor is difficult to determine given the likelihood that clone 10 RNA is not inducible by glucocorticoids before the adipogenic conversion. Thus, differentiation is accompanied by some change that confers hormone responsiveness to the clone 10 gene. This change is not mediated by the induction of previously absent receptors, as both 10T1/2 and TA1 preadipocytes have functional glucocorticoid receptors. Rather, differentiation may lead to an alteration of chromatin structure or generation of a transcription factor(s) that allows clone 10 induction and expression.

Recent studies on mouse mammary tumor virus have revealed a correlation between gene activity and hormonal inducibility which is reflected in an altered chromatin DNase sensitivity (14, 50). Studies of the chicken vitellogenin gene have revealed DNase hypersensitive sites that exist before estrogen-mediated expression and that correlate in tissue distribution with its hormone inducibility (6). It has not yet been possible, though, to observe and study the generation of such sites that mark future hormone inducibility during differentiation. TA1 cells may offer an advantage in characterizing such changes since the precursor cells in which clone 10 RNA is not inducible are readily available. Analysis of clone 10 chromatin structure is underway in an attempt to find changes that correlate with transcriptional activity, potential hormone inducibility, and hormone induction itself. If the induction of clone 10 transcription is indeed a primary glucocorticoid response it will be particularly interesting to identify DNA binding sites for the glucocorticoid-receptor complex and

analyze the factors that affect their chromatin structure during differentiation.

We thank Dr. H. Blau for her careful reading of the manuscript, P. Gunning and L. Kedes for the β -actin cDNA, and Karen Benight for excellent secretarial assistance.

This work was supported by grants from the March of Dimes and the National Institutes of Health. A. B. Chapman is supported by a National Institutes of Health Medical Scientist Training Grant to Stanford Medical School. D. M. Knight is supported by a National Institutes of Health postdoctoral fellowship. G. M. Ringold is an Established Investigator of the American Heart Association.

Received for publication 1 April 1985, and in revised form 28 May 1985.

REFERENCES

1. Ailhaud, G. 1982. Adipose cell differentiation in culture. *Mol. Cell. Biochem.* 49:17-31.
2. Ashburner, M., C. Chihara, P. Meltzer, and G. Richards. 1973. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 38:655-662.
3. Avery, M. E. 1975. Pharmacologic approaches to the acceleration of fetal lung maturation. *Br. Med. Bull.* 31:13-17.
4. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidyl acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408-1414.
5. Ballard, P. L., J. D. Baxter, J. J. Higgins, G. G. Rousseau, and G. M. Tomkins. 1974. General presence of glucocorticoid receptors in mammalian tissues. *Endocrinology.* 94:998-1002.
6. Burch, J. B. E., and H. Weintraub. 1983. Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. *Cell.* 33:65-76.
7. Chapman, A. B., D. M. Knight, B. S. Dieckmann, and G. M. Ringold. 1984. Analysis of gene expression during differentiation of adipogenic cells in culture and hormonal control of the developmental program. *J. Biol. Chem.* 259:15548-15555.
8. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294-5299.
9. Coore, H. G., R. M. Denton, B. R. Martin, and P. J. Randle. 1971. Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. *Biochem. J.* 125:115-127.
10. Cox, R. F. 1973. Transcription of high-molecular-weight RNA from hen-oviduct chromatin by bacterial and endogenous form-B RNA polymerases. *Eur. J. Biochem.* 39:49-61.
11. Dente, L., G. Cesareni, and R. Cortese. 1983. pEMBL: a new family of single stranded plasmids. *Nucleic Acids Res.* 11:1645-1655.
12. Fain, J. N., and M. P. Czech. 1975. Glucocorticoid effects on lipid mobilization and adipose tissue metabolism. In *Handbook of Physiology, Section 7. Endocrinology, Volume VI, Adrenal Gland.* H. Blaschko, G. Sayers, and A. D. Smith, editors. American Physiological Society, Washington, D.C. 169-178.
13. Fain, J. N., V. P. Kovacev, and R. O. Scow. 1965. Effects of growth hormone and dexamethasone on lipolysis and metabolism in isolated fat cells of the rat. *J. Biol. Chem.* 240:3522-3529.
14. Feinstein, S. C., S. R. Ross, and K. R. Yamamoto. 1982. Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. *J. Mol. Biol.* 156:549-565.
15. Frieden, E., and J. J. Just. 1970. Hormone responsiveness in amphibian metamorphosis. In *Biochemical Action of Hormones, Volume 1.* G. Litwack, editor. Academic Press, Inc., New York. 1-52.
16. Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark. 1984. Transcriptional and post-transcriptional regulation of interferon-induced gene expression in human cells. *Cell.* 38:745-755.
17. Green, H., and O. Kehinde. 1975. An established preadipocyte cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell.* 5:19-27.
18. Israel, D. J., and J. P. Whitlock, Jr. 1984. Regulation of cytochrome P₁-450 gene transcription by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in wild type and variant mouse hepatoma cells. *J. Biol. Chem.* 259:5400-5402.
19. Jost, A., and L. Picon. 1970. Hormonal control of fetal development and metabolism. *Adv. Metab. Disord.* 4:123-184.
20. Karin, M., R. D. Anderson, E. Slater, K. Smith, and H. R. Herschman. 1980. Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction process. *Nature (Lond.)* 286:295-297.
21. Lai, E., O. M. Rosen, and C. S. Rubin. 1982. Dexamethasone regulates the β -adrenergic receptor subtype expressed by 3T3-L1 preadipocytes and adipocytes. *J. Biol. Chem.* 257:6691-6696.
22. Lawrence, J. C., and J. Larner. 1978. Activation of glycogen synthase in rat adipocytes by insulin and glucose involves increased glucose transport and phosphorylation. *J. Biol. Chem.* 253:2104-2113.
23. Liggins, G. C. 1976. Adrenocortical-related maturational events in the fetus. *Am. J. Obstet. Gynecol.* 126:931-939.
24. Malinowska, K. W., R. N. Hardy, and P. W. Nathanielsz. 1972. Neonatal adrenocortical function and its possible relation to the uptake of macromolecules by the small intestines of guinea pig and rabbit. *J. Endocrinol.* 55:397-404.
25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, NY. 202-203.
26. Margolis, F. L., J. Roffi, and A. Jost. 1966. Norepinephrine methylation in fetal rat adrenals. *Science (Wash. DC.)* 154:275-276.
27. McKnight, G. S., and R. D. Palmiter. 1979. Transcriptional regulation of the ovalbumin

- and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.* 254:9050-9058.
28. Morikawa, M., T. Nixon, and H. Green. 1982. Growth hormone and the adipose conversion of 3T3 cells. *Cell.* 29:782-789.
 29. Murphy, B. E. P., and C. T. L. Branchaud. 1983. Fetal metabolism of cortisol. In *Current Topics in Experimental Endocrinology*. Volume 5. L. Martini and V. H. T. James, editors. Academic Press, Inc., New York. 198-229.
 30. Paddington, R., and A. A. Moscona. 1967. Precocious induction of retinal glutamine synthetase by hydrocortisone in the embryo and in culture. Age-dependent differences in tissue response. *Biochim. Biophys. Acta.* 141:429-432.
 31. Renkawitz, R., G. Schutz, D. Von der Ahe, and M. Beato. 1984. Sequences in the promoter region of the chick lysozyme gene required for steroid regulation and receptor binding. *Cell.* 37:503-510.
 32. Reznikoff, C., D. Brankow, and C. Heidelberger. 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to post confluence inhibition of division. *Cancer Res.* 33:3231-3238.
 33. Rigby, P. W. J., M. M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
 34. Ringold, G. M. 1985. Steroid hormone regulation of gene expression. *Annu. Rev. Pharmacol.* 25:529-566.
 35. Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varmus. 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. *Proc. Natl. Acad. Sci. USA.* 74:2879-2883.
 36. Ringold, G. M., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus. 1975. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. *Cell.* 6:299-305.
 37. Rosen, J., and B. W. O'Malley. 1975. Hormonal regulation of specific gene expression in the chick oviduct. In *Biochemical Action of Hormones*. Volume 3. G. Litwack, editor. Academic Press, Inc., New York. 271-315.
 38. Rosen, O. M., C. J. Smith, A. Hirsch, E. Lai, and C. S. Rubin. 1979. Recent studies of the 3T3-L1 adipocyte-like cell line. In *Recent Progress in Hormone Research*. Volume 35. R. O. Greep, editor. Academic Press, Inc., New York. 477-499.
 39. Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen. 1978. Development of hormone receptors and hormonal responsiveness *in vitro*. *J. Biol. Chem.* 253:7570-7578.
 40. Russell, T. R., and R. Ho. 1976. Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F_{2α} and 1-methyl-3-isobutyl-xanthine. *Proc. Natl. Acad. Sci. USA.* 73:4516-4520.
 41. Schimke, R. T., G. S. McKnight, and D. J. Shapiro. 1975. Nucleic acid probes and analysis of hormone action in oviduct. In *Biochemical Action of Hormones*. Volume 3. G. Litwack, editor. Academic Press, Inc., New York. 245-269.
 42. Smith, M. M., A. E. Reene, and R. C. C. Huang. 1978. Analysis of RNA initiated in isolated mouse myeloma nuclei using purine nucleoside 5'(γ -S) triphosphates as affinity probes. *Cell.* 15:615-626.
 43. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201-5205.
 44. Vannice, J. L., J. R. Grove, and G. M. Ringold. 1983. Analysis of glucocorticoid-inducible genes in wild type and variant rat hepatoma cells. *Mol. Pharmacol.* 23:779-785.
 45. Vannice, J. L., J. M. Taylor, and G. M. Ringold. 1984. Glucocorticoid-mediated induction of α_1 -acid glycoprotein: evidence for hormone-regulated RNA processing. *Proc. Natl. Acad. Sci. USA.* 81:4241-4245.
 46. Williams, I. H., and S. E. Polakis. 1977. Differentiation of 3T3-L1 fibroblasts to adipocytes: the effects of indomethacin, prostaglandin E₁, and cyclic AMP on the process of differentiation. *Biochem. Biophys. Res. Commun.* 77:175-186.
 47. Wise, L. S., and H. Green. 1979. Participation of one isozyme of cytosolic glyceraldehyde dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.* 254:273-275.
 48. Wu, G. J., and G. Zubay. 1974. Prolonged transcription in a cell-free system involving nuclei and cytoplasm. *Proc. Natl. Acad. Sci. USA.* 71:1803-1807.
 49. Wyatt, G. R. 1972. Insect Hormones. In *Biochemical Action of Hormones*. Volume 2. G. Litwack, editor. Academic Press, Inc., New York. 386-490.
 50. Zaret, K. S., and K. R. Yamamoto. 1984. Reversible and persistent changes in the chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. *Cell.* 38:29-38.