Steroid Hormone Regulation of Ovalbumin and Conalbumin Gene Transcription

A MODEL BASED UPON MULTIPLE REGULATORY SITES AND INTERMEDIARY PROTEINS*

(Received for publication, January 26, 1981)

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Changes in rates of ovalbumin and conalbumin gene transcription and mRNA levels were monitored during an entire cycle of estrogen withdrawal and restimulation. Correlations of transcription rates with nuclear estrogen receptor levels in this experiment and in dose-response experiments reveal that conalbumin gene transcription is directly proportional to nuclear receptor levels, whereas ovalbumin gene transcription is related to receptor levels in a way that suggests cooperative interactions among receptors. Conalbumin mRNA accumulation and transcription are transiently inhibited by administration of progesterone to estrogen-stimulated chicks, whereas ovalbumin gene transcription is stimulated by this regimen. This puzzling observation can be rationalized if a single receptor binding site is involved in conalbumin gene regulation and multiple sites are involved in ovalbumin gene regulation. These ideas are combined with our recent observations that protein synthesis inhibitors and butyrate selectively, but reversibly, inhibit ovalbumin and conalbumin gene transcription. We describe a new hypothesis of how steroid hormones regulate egg white gene transcription in the chick oviduct.

Steroid hormones modulate mRNA levels in a wide variety of systems (1–3). Most often, specific mRNA levels are increased in response to steroid hormones; but in some cases, mRNA levels decline (4, 5). Changes in mRNA abundance have been shown in a few cases to be mediated by changes in the rate of mRNA transcription (6–9), although mRNA stability may also be affected (9). The mechanisms by which steroid hormones mediate mRNA transcription and stability are unknown, but it is generally assumed that transcriptional regulation is mediated by nuclear steroid receptors that bind to specific sites near the genes they regulate. The challenge in the field of steroid hormone action is to characterize these postulated receptor binding sites and define the molecular connection between receptor binding and transcriptional activation (or inactivation in certain systems).

We have been studying the regulation of ovalbumin and conalbumin mRNA production in the chick oviduct by steroid hormones. The accumulation of mRNA, and mRNA, can be induced in the same tubular gland cells in response to any of four different classes of steroid hormones (estrogens, progestins, glucocorticoids, and androgens), each acting through a distinct cellular receptor (10–13). We have noted significant differences in the kinetics of mRNA, and mRNA, accumulation in response to different doses of these steroids and combinations of them (12, 13). We assume that these genes respond to each of the steroids by similar mechanisms; thus, the differences in response provide valuable clues about the nature of these mechanisms. In this paper, we extend our analysis by correlating rates of ovalbumin and conalbumin gene transcription with nuclear receptor levels. Transcription was monitored by allowing RNA polymerases that had initiated in vivo under various hormonal conditions to elongate RNA chains in vitro in the presence of radioactive precursors, and then quantitating the relative number of specific gene transcripts by hybridizing the RNA to immobilized plasmids containing the DNA of interest (9). This assay essentially measures the steady state number of polymerases on the gene of interest, and hence, it is independent of hormonal effects on mRNA processing or stability. Since steroid receptor levels can be measured in the same nuclear preparations used for transcription analysis, these assays provide the most direct correlation between steroid hormone receptors and transcriptional activation devised to date. Based upon the results of these studies and the effects of selective inhibitors of transcription (14), we present a simple model that accounts for the differential transcriptional response of the ovalbumin and conalbumin genes. This model is considerably different than one proposed 5 years ago (2) and reflects the refinement of our ideas made possible by direct analysis of transcription.

MATERIALS AND METHODS

Procedures for administering hormones (15) measuring nuclear estrogen receptors (16), protein synthesis (1), mRNA levels (17), and specific gene transcription (9) have been described; supplementary details are included in the figure legends.

RESULTS

Kinetic Relationships between Nuclear Receptors and Transcription—Fig. 1 shows the results of an experiment in which chicks that had been on secondary stimulation with subcutaneous diethylstilbestrol implants (15) were withdrawn by removal of the implants, and 36 h later, the chicks were restimulated by daily injection of 17β-estradiol benzoate. Samples were taken throughout this time course to measure: (a) mRNA, and mRNA, levels by cDNA hybridization, (b) mRNA, ovalbumin mRNA; mRNA, conalbumin mRNA.

* This work was supported by Grant HD-09172 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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were pooled; aliquots were used to measure mRNA levels (○), transcription (histograms) and nuclear receptor levels (▲). A, ovalbumin gene transcription and mRNA levels; B, conalbumin gene transcription and mRNA levels; C, nuclear estrogen receptor levels. The transcription reaction mixtures were as described (9), except that creatine phosphate, creatine phosphokinase, and nucleotide diphosphate kinase were also included to help maintain the phosphorylation of [α-32P]UTP (18). Using these conditions, 1 to 3 × 10^6 cpm of [32P]RNA were synthesized in a 100-μl reaction mixture containing 4 × 10^7 nuclei (100 μg of DNA). Three different concentrations of [32P]RNA were hybridized to sets of three filters containing immobilized ovalbumin cDNA plasmid, conalbumin cDNA plasmid, or pBR322; [3H]RNA standards were included in each reaction to monitor and correct for the hybridization efficiency which ranged from 10 to 40%. Details are provided in Ref. 9.

Fig. 1. Effects of estrogen withdrawal and readministration upon transcription, mRNA, and nuclear receptor levels. Chicks that had been maintained on two 15-mg diethylstilbestrol pellets for 10 days were withdrawn by removing the pellets at time 0. Starting at 36 h, the remaining chicks were injected daily (see arrows) with estradiol benzoate (1 mg in 0.4 ml of corn oil). At the indicated times, groups of three chicks were killed and the magnum portions of the oviducts were pooled; aliquots were used to measure mRNA levels (○), transcription (histograms) and nuclear receptor levels (▲). A, ovalbumin gene transcription and mRNA levels; B, conalbumin gene transcription and mRNA levels; C, nuclear estrogen receptor levels. The transcription reaction mixtures were as described (9), except that creatine phosphate, creatine phosphokinase, and nucleotide diphosphate kinase were also included to help maintain the phosphorylation of [α-32P]UTP (18). Using these conditions, 1 to 3 × 10^6 cpm of [32P]RNA were synthesized in a 100-μl reaction mixture containing 4 × 10^7 nuclei (100 μg of DNA). Three different concentrations of [32P]RNA were hybridized to sets of three filters containing immobilized ovalbumin cDNA plasmid, conalbumin cDNA plasmid, or pBR322; [3H]RNA standards were included in each reaction to monitor and correct for the hybridization efficiency which ranged from 10 to 40%. Details are provided in Ref. 9.

the instantaneous rate of transcription of ovalbumin and conalbumin genes in isolated nuclei, and (c) the number of nuclear estrogen receptors.

A steady state level of about 4500 nuclear receptors/cell was achieved with either estrogen source. Removal of the diethylstilbestrol pellets led to a rapid loss of receptors from the nuclei; after 36 h, a new steady state level of ~200 nuclear receptors/cell was achieved (Fig. 1C). This decline in nuclear receptors was accompanied by a decline in transcription rates and mRNA levels. Conalbumin gene transcription fell in parallel with the loss of nuclear receptors; ovalbumin gene transcription fell more rapidly. When estradiol benzoate was administered, transcription from the conalbumin gene recovered more rapidly than from the ovalbumin gene. As expected, mRNA levels increased more slowly than transcription rates. Complete recovery of all parameters was achieved by 64 h after restimulation. It is important to note that with this experimental regimen the cell population (~50% tubular gland cells) was essentially unchanged throughout the time course (15); although there was a 2- to 3-fold loss of magnum wet weight during estrogen withdrawal that was regained in 2 to 3 days.

The experiment shown in Fig. 1 and previous experiments (9) indicate that recovery of nuclear receptors and transcription rates was not instantaneous upon administration of 1 mg of estradiol benzoate to birds withdrawn for more than 1 day; in fact, full response was not achieved for several days. To explore the kinetics of this loss of responsiveness, birds were injected with 1 mg of estradiol benzoate at various times during the withdrawal process and 6 h later nuclear receptors, transcription rates, and mRNA levels were determined. Fig. 2 shows the results of these "estrogen-challenge" experiments superimposed upon the withdrawal data shown in Fig. 1. At early times of withdrawal (<12 h), estrogen challenge to this all of the receptors back into the nucleus and transcription rates are restored, but at later times there is a progressive loss of the ability of estrogen to restore nuclear receptors and transcription. This effect is most obvious on mRNA, synthesis (Fig. 2). We conclude that during estrogen withdrawal the oviduct becomes refractory to maximal restimulation.2 The decline in nuclear estrogen receptors triggers many catabolic events including mRNA destabilization, protein degradation, and decreased translational efficiency (15, 19). This catabolic state may also lead to a loss of total estrogen receptors, and hence a decline in the number that can be mobilized back into the nucleus upon estrogen challenge. Complete recovery takes 24 h (Fig. 1) and presumably depends upon receptor synthesis.

The experiment shown in Fig. 2 reveals an apparent disso-
ciation between mRNA synthesis and mRNA accumulation. When oviducts that were in the middle phase of withdrawal (between 12 and 18 h) were challenged with estrogen, nuclear receptors and transcription rates increased while mRNA, levels continued to decline. Two factors contribute to this result. First, it is at least partially a consequence of the fact that the rate of transcription from the ovalbumin gene falls faster than mRNA, levels and transcription rates are not fully restored within 6 h; thus, during the 6-h challenge, mRNA, continues to approach a level dictated by the average rate of transcription during the interval. Second, mRNA catabolism is triggered by loss of receptors from the nucleus (15) and may not be readily reversed during the middle phase of withdrawal (19); hence, mRNA levels may fall because the rate of mRNA degradation is greater than in the presence of optimal estrogen.

The relationship between nuclear receptor levels and rates of transcription is shown more clearly in Fig. 3 which combines all of the data from Figs. 1 and 2. When plotted in this form, it is apparent that conalbumin gene transcription is directly related to nuclear receptor levels in a more complex way. Note that when nuclear receptors are half-

2 The dose of 1 mg of estradiol benzoate was chosen for these experiments because previous studies indicated that this dose produced maximum stimulation of mRNA, accumulation in birds withdraw-

for more than 10 days (16). However, results presented in Fig.

4 indicate that this dose is inadequate after 2 days of withdrawal. Nevertheless, even with an optimal dose of 5 mg of estradiol benzoate, maximal nuclear receptor levels and transcription rates are not achieved within 8 h (see Fig. 4).
maximal, the rate of mRNA\textsubscript{ov} transcription is only 12% of maximum and only when receptors reach 90% of maximum does mRNA\textsubscript{ov} transcription become half-maximal. We suggested previously (9), that two or more receptor binding sites may be involved in regulating ovalbumin gene transcription such that the rate of ovalbumin gene transcription is proportional to \( R^n \), where \( R \) is the fractional saturation of the nucleus with receptors and \( n \) is the number of sites. In effect, this equation predicts that transcription rates are related to the probability of filling \( n \) sites and that only when all sites are filled is transcription activated; e.g. when \( n = 2 \) and receptors are half-maximal, the rate of transcription would be \((0.5)^5 = 25\% \) of maximum. The dotted lines on Fig. 3 are theoretical curves for the above equation with \( n = 2, 3, \) and 5. It is clear that this type of equation is too simple to describe the relationship between nuclear receptors and mRNA\textsubscript{ov} synthesis. The dashed line, which fits the data better, is derived from a polynomial expression in which the relative rate of transcription equals 0.15 \( R + 0.85 R^2 \). Polynornial expressions with less than 5th order terms or with positive values for \( R^2 \), \( R^3 \), or \( R^4 \) do not fit the data as well. This formulation suggests that there are five receptor binding sites and that filling one site allows a low level of transcription (~15% of maximum), filling additional sites has little or no effect until the fifth site is filled which then allows maximal transcription rates.

Dose-Response Relationships—The same type of relationship between nuclear receptors and transcription rates is observed when different dosages of estrogen are administered to chicks withdrawn from estrogen for 33 h. Fig. 4A shows the results obtained after 8 h of secondary stimulation with 20 \( \mu \)g to 8 mg of estradiol benzoate. Synthesis of mRNA\textsubscript{ov} increases in parallel with nuclear estrogen receptor levels and is half-maximal at 370 \( \mu \)g of estradiol benzoate per bird, whereas mRNA\textsubscript{ov} synthesis is clearly displaced relative to nuclear receptors and is half-maximal with 1000 \( \mu \)g of estradiol benzoate. After long term withdrawal (>10 days) from estrogen, about 3.5-fold less steroid is required for comparable responses (16). The most likely explanation for this disparity is that during early stages of withdrawal, the concentration of "free" steroid in the serum is considerably lower than it is after long term withdrawal due to either a greater rate of metabolic clearance or a higher concentration of estrogen-binding pro-

![Fig. 2. Restimulation of nuclear receptors, transcription, and mRNA accumulation during withdrawal. Some of the chicks that were in the process of withdrawal from estrogen were restimulated with 1 mg of estradiol benzoate for 6 h and assayed as in Fig. 1 (solid symbols). Open symbols represent data taken from Fig. 1. A, ovalbumin mRNA levels; B, conalbumin mRNA levels; C, ovalbumin gene transcription; D, conalbumin gene transcription; and E, nuclear estrogen receptor levels.](image)

![Fig. 3. Correlation of nuclear estrogen receptor levels with rates of ovalbumin and conalbumin gene transcription. Data from Figs. 1 and 2 are plotted to show the relationship of each gene to nuclear receptor levels. The solid and dotted lines are theoretical curves based on the equation: \( T_i = R^n \) where \( T_i \) is the fractional rate of transcription, \( R \) is the fractional nuclear receptor concentration, and \( n \) is the number of binding sites. The dashed line is the theoretical curve generated by the equation \( T_i = 0.15R + 0.85R^2 \) (see text).](image)

![Fig. 4. Dose-response relationship of conalbumin and ovalbumin gene transcription and nuclear estrogen receptor levels. Chicks withdrawn from diethylstilbestrol pellets for 35 h were injected with 0.4 ml of corn oil containing the indicated amounts of 17β-estradiol benzoate and killed 8 h later. A, relative rate of ovalbumin (○) and conalbumin (●) transcription and the level of nuclear estrogen receptors (□). B, correlation of receptors with transcription rates. The line through the conalbumin data is simply \( T_i = R_i \) whereas the curve through the ovalbumin data is based on the equation \( T_i = 0.15R + 0.85R^2 \) (see text).](image)
teins in the serum. In Fig. 4B the dose-response data are plotted to reveal the relationship between nuclear receptor levels and rates of transcription. The results are very similar to those shown in Fig. 3 and the lines drawn are derived using the equations described above.

**Interaction of Estrogen and Progesterone Receptors at the Transcriptional Level**—We have shown previously that the early kinetics of induction of conalbumin synthesis are remarkably different with estrogen or progesterone (2, 12, 16). With estrogen, conalbumin synthesis is induced immediately, whereas with progesterone, there is a lag of ~2 h and then the rate of synthesis increases slowly over the next few hours (2). When estrogen and progesterone are administered together, progesterone is apparently dominant since the kinetics of conalbumin synthesis resemble those with progesterone alone (20). Moreover, this dominance is apparent even when progesterone is administered several hours after estrogen; in this situation, estrogen-induced conalbumin synthesis is abruptly inhibited but then gradually recovers after a few hours (20, 21). The most puzzling feature of this phenomenon is that ovalbumin synthesis is stimulated at the same time that conalbumin synthesis is inhibited, suggesting that there is some fundamental difference in the way receptors interact at sites involved in the regulation of these genes.

To explore this phenomenon further, we measured the dose of progesterone required for the inhibition of conalbumin synthesis and the stimulation of ovalbumin synthesis. Fig. 5 shows the results of an experiment in which either 1 mg of estradiol benzoate or 2 mg of progesterone were administered alone for 4 h (to establish the boundaries) or varying amounts of progesterone were administered along with 1 mg of estradiol benzoate. Half-maximal inhibition of conalbumin synthesis required slightly more progesterone (100 µg) than half-maximal stimulation of ovalbumin synthesis (65 µg). The amount of progesterone required for these effects is in the same range as is required when acting alone, but the relative sensitivity of the two genes to progesterone is reversed; since, when acting alone, about twice as much progesterone is required to induce ovalbumin synthesis half-maximally as compared to conalbumin synthesis (16).

The experiment shown in Fig. 6 reveals the effect of delayed progesterone administration on the kinetics of estrogen-induced mRNA accumulation and transcription. Progesterone has an immediate inhibitory effect on mRNA<sub>con</sub> accumulation (Fig. 6B) and a synergistic effect on the mRNA<sub>ov</sub> accumulation (Fig. 6C), in agreement with earlier protein synthesis data (20). The effects of progesterone on specific gene transcription rates are consistent with the mRNA accumulation results. Conalbumin gene transcription falls immediately after progesterone administration and then gradually recovers (Fig. 6D), while ovalbumin gene transcription increases 2-fold (Fig. 6E). The administration of progesterone to estrogen-treated chicks results in a transient loss of nuclear estrogen receptors as shown in Fig. 6A. Nuclear estrogen receptor levels reach minimal values about 2 to 4 h after progesterone administration and then gradually return to normal levels (21).

It is important to note that all of the experiments described in this section were conducted with birds withdrawn from primary estrogen stimulation for 10 days or more; hence, the

![Fig. 5. Concentration of progesterone required for simultaneous inhibition of estrogen-induced conalbumin synthesis and stimulation of ovalbumin synthesis. Chicks withdrawn for 11 days were administered 2 mg of progesterone alone (C), or 1 mg of estradiol benzoate plus varying amounts of progesterone (D), and 4 h later, oviducts were isolated and incubated for 30 min in Hanks' salts plus [14C]leucine (15 µCi/ml). The incorporation of [14C]leucine into (A) conalbumin and (B) ovalbumin was determined immunologically.](image)

![Fig. 6. Effect of delayed progesterone administration on estrogen-induced transcription of conalbumin and ovalbumin genes. Chicks withdrawn from estrogen for 13 days were administered 1 mg of estradiol benzoate at time zero, and 2 h later, half of the chicks received 1 mg of progesterone. Oviducts were isolated at the times indicated and used to measure: (A) nuclear estrogen receptors, (B) accumulation of mRNA<sub>con</sub>, (C) accumulation of mRNA<sub>ov</sub>, (D) relative rate of mRNA<sub>con</sub> synthesis, and (E) relative rate of mRNA<sub>ov</sub> synthesis. (F) estrogen treatment only; (G) estrogen treatment plus progesterone at 2 h.](image)
concentration of estradiol benzoate used (1 mg) was optimal (16) but the percentage of responsive cells was 3- to 4-fold lower than in the studies described in the previous sections (15). Moreover, the simultaneous inhibition of mRNA acc. accumulation and stimulation of mRNA acc. accumulation by progesterone has been reproduced in oviduct explants in culture (17) indicating that this phenomenon is a property of these hormones acting directly on the oviduct. However, when identical experiments were performed with birds withdrawn for only 2 days, mRNA acc. accumulation and transcription were stimulated, nuclear estrogen receptor levels fell, but mRNA acc. accumulation and transcription were unaffected (data not shown). These results were also reproduced in culture, indicating that the progesterone-mediated inhibition of mRNA acc. production changes with the duration of withdrawal and is a property of the oviduct cells rather than of serum components.

The effect of progesterone on transcription and nuclear estrogen receptors has also been explored in long term, estrogen-stimulated chicks. In this case, progesterone transiently inhibits both mRNA acc. and mRNA acc. synthesis (Fig. 7). Corresponding mRNA levels are affected only slightly (data not shown) as would be expected since there is a high concentration of both mRNAs in a fully stimulated oviduct and they have relatively long half-lives. Progesterone also depresses nuclear estrogen receptor levels transiently (Fig. 7). Note that transcription rates recover more rapidly than nuclear estrogen receptors.

**Discussion**

Although it has generally been assumed that steroid hormones act in the nucleus to modulate mRNA synthesis (1–3, 22, 23), direct correlations between nuclear receptor levels and rates of transcription of specific genes have only recently become possible (9, 24). The kinetic analyses of transcriptional activation of two egg white genes by estrogen and progesterone that are presented here are consistent with earlier measurements of ovalbumin and conalbumin mRNA accumulation and confirm that these genes are not regulated identically. Both genes show features that were not initially anticipated in simple models of hormonal activation of transcription.

The salient features of transcriptional regulation of these oviduct genes that require explanation are indicated below:

(a) Transcription of the conalbumin gene is activated as rapidly as estrogen receptors reach the nucleus, whereas transcription of the ovalbumin gene is activated considerably slower (Figs. 1 and 6 and Ref. 9). (b) Conalbumin gene transcription is directly proportional to nuclear estrogen receptor concentration whereas ovalbumin gene transcription shows an exponential relationship to receptor concentration (Figs. 3 and 4). (c) Estrogen and progesterone receptors act synergistically on ovalbumin gene transcription during early stages of induction (Fig. 6) but not after full activation (Fig. 7). (d) In contrast, progesterone receptors have a transient inhibitory effect on estrogen-induced conalbumin gene transcription in fully withdrawn or fully stimulated oviducts (Figs. 6 and 7), but not in partially withdrawn oviducts. (e) Transcription of both genes is prevented by protein synthesis inhibitors and by inhibitors of histone deacetylation such as butyrate (14). Neither class of drugs has obvious effects on overall transcription rates or nuclear receptor levels. If butyrate or inhibitors of protein synthesis are added to cells after induction by steroids, then specific transcription falls rapidly (14).

This complex regulation is distinct from the relatively simple regulation of murine mammary tumor virus expression (6, 7) or metallothionein genes by glucocorticoid hormones (25) or Drosophila "early" genes by ecdysone (26, 27).

One simple model that accounts for the hormonal regulation of ovalbumin and conalbumin genes is shown in Fig. 8. Basically, the model predicts that a single receptor binding site is involved in the regulation of the conalbumin gene whereas there are several sites involved in ovalbumin gene regulation. Each of the four classes of active steroid receptors (estrogen, progesterin, androgen, and glucocorticoid) is assumed to bind to the same sites, but the affinities may vary. For both genes, receptor binding is coupled to transcriptional activation by one or more intermediary proteins with short half-lives. Thus, inhibition of protein synthesis leads to a rapid inhibition of hormonally induced transcription (14, 17). The intermediary proteins may be subject to covalent modification, a process that reversibly activates and inactivates their capacity to convert receptor binding into transcriptional activation. This latter aspect of the model is included to account for the reversible inhibition of egg white gene transcription by butyrate (14). Covalent modification of receptors or adjacent nucleosomes would be equally plausible sites of butyrate control. The covalent modification is likely to be acetylation but might involve other modifications as well.

The prediction of multiple receptor binding sites for the ovalbumin gene compared to a single site for the conalbumin gene readily accounts for the kinetic and dose-response relationships because it would take longer to fill multiple sites and because transcription of a gene with multiple sites would not be expected to show direct proportionality with nuclear receptor levels. The experiments described here are not precise enough to define the nature of the receptor interactions within the multiple binding sites. However, the present data suggest that filling one site allows partial activation of the ovalbumin gene, but filling more sites has little effect until all of the sites are filled. A physical interpretation of this type of model is that the last receptor to bind provides a stable, possibly symmetrical, conformation of chromatin near the ovalbumin gene promoter. For example, the steroid receptors may bind as a ring around the chromatin fiber rather than along the fiber as envisaged in Fig. 8.

Nuclear estrogen receptors are clearly rate-limiting for both conalbumin and ovalbumin gene transcription. Maximum

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Figure 7: Effect of progesterone on nuclear estrogen receptors and specific gene transcription in long term, estrogen-stimulated chicks. Chicks maintained on estrogen were injected with 1 mg of progesterone. At various times, chicks were killed and oviduct samples were used to measure the number of nuclear estrogen receptors (●) and the relative rate of mRNA acc. (open histograms) and mRNA acc. synthesis (shaded histograms).

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Further understanding of the molecular details of hormonal induction in this system requires identification of the DNA sequences responsible for steroid receptor activation of transcription. The putative 5' regulatory regions of both the ovalbumin and conalbumin genes have been cloned and large regions have been sequenced (31–33) although receptor binding sites have not yet been identified. The recent observations that transcription from these cloned genes is faithfully reproduced in a cell-free system is encouraging (34, 35); however, reconstitution of transcriptional regulation may be considerably more difficult considering the complexities described above.

Acknowledgments—We thank our colleagues for many helpful discussions during the course of this work. We appreciate the secretarial assistance of Abby Dudley.

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