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STEROID HORMONE REGULATION OF GENE EXPRESSION

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INTRODUCTION

The remarkable diversity in form and function of cells within a single organism stems not from intrinsic differences in genetic composition but from the selective and highly controlled expression of subsets of that genetic repertoire. A major focus of current biological research is the elucidation of the mechanisms by which gene expression can be regulated both during development and in response to environmental stimuli. It is the ability of steroid hormones to act as gene regulatory molecules that has focused tremendous attention on their mode of action.

Each class of steroid hormone appears to mediate its biological response by binding to an intracellular receptor protein that is confined to target tissues [reviewed in (1, 2)]. Interaction of the hormone with its cognate receptor leads to an alteration in the structure of the protein that is manifested by an increased affinity of the steroid-receptor (SR) complex for DNA [reviewed in (3)]. By analogy with other ligand-protein interactions, the role of the hormone itself can best be viewed as an allosteric modifier of receptor structure. One exception to this general model is the membrane receptor for progesterone found on *Xenopus* oocytes (4).

Although SR complexes bind to all DNAs, albeit with low binding constants, it is their high-affinity interactions with specific DNA sequences that seem to be important for the ensuing alterations in gene expression. As a result, only a small number of genes within the target cell become transcriptionally activated. It is my intent in this review to summarize key observations from the

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large body of work that has led to our current understanding of steroid receptor structure and function as it pertains to activation of gene transcription. In addition, I will briefly introduce novel modes by which steroids effect changes in gene expression. It is through a concerted interplay of gene regulatory events, in large part (if not completely) initiated by direct stimulation of transcription by SR complexes, that predictable and reproducible effects on cell function transpire. Particular attention will be paid to systems that have been and those that have promise of being especially useful in deciphering the intricacies of steroid receptor action. The reader will be directed to other sources for more detailed discussions of receptor structure, modification, and activation, as well as for additional information pertaining to specific systems.

THE EVOLUTION OF THE MODEL OF STEROID RECEPTOR ACTION

Although perhaps not generally appreciated, some of the earliest studies involving detection of receptors with radioactive ligands were performed by Jensen & DeSombre in the course of their studies on estrogen-responsive tissues (1). They observed that ^{3}H estradiol accumulated in tissues such as uterus that are known to exhibit marked physiological changes in response to hormone treatment. Based on these observations, Jensen postulated the existence of highly specific receptors for estradiol in these target tissues. Subsequent analysis of the estradiol receptor by Jensen's and Gorski's groups led to the observation that the unoccupied receptor resides primarily in the cytoplasm, whereas after hormone treatment the bulk of the receptor becomes tightly associated with the nucleus (5, 6). Similar data were later obtained for other classes of steroids (2). The proposed two-step model of steroid hormone action was widely accepted and as postulated suggested that the hormone causes net migration of receptor protein from the cytosolic to the nuclear compartment. Since it was clear that steroids cause alterations in the production of specific proteins in various systems (see below), the idea that the nuclear form of the SR complex regulates gene expression arose [for reviews see (7, 8)]. The observation that the receptor could bind to DNA in vitro, but only after binding to the hormone, added significantly to the model (9, 10).

Recent technological developments have provided powerful new approaches to the study of steroid receptors. As a result of such studies, the classical two-step model has had to undergo minor modifications. First, novel methods for fractionating cells into nuclear and cytoplasmic fractions led to the observation that unoccupied estrogen receptors reside primarily, if not completely, within the nucleus (11, 12). Second, immunocytochemistry with monoclonal antibodies against the human estrogen receptor also indicate that both the naive and ligand-bound forms of the receptor are localized within the nucleus (13).

Thus, the alteration of receptor structure and/or function associated with binding of hormone could reflect a redistribution of the receptor from a non–DNA containing nuclear compartment to the chromosomal DNA. The earlier studies on receptor localization were undoubtedly compromised by the fact that, upon lysing cells in large aqueous volumes, receptors appear to dissociate from nuclear components. Similar observations have been made for unrelated proteins such as α DNA polymerase (14) and the dioxin receptor (15). In the case of two steroid hormones, ecdysone and Vitamin D, as well as that of thyroxine, the unoccupied form of their corresponding receptors remains with the nuclear fraction even under harsh disruption conditions [reviewed in (16)]. The nature of the sites to which any or all of these receptors bind in the absence of hormone remains obscure. However, Barrack & Coffey (17) have suggested that estrogen receptors may be associated with a rather ill-defined but increasingly provocative structure composed of DNA and protein that they have called the nuclear matrix. Since both DNA replication and transcription machinery may be associated with this structure, Gorski (18) has raised the intriguing possibility that steroid receptors might remain immobilized on the nuclear matrix; the hormonal activation of the receptor would then be envisioned to expose a DNA binding site that would cause the SR complex to become tightly associated with DNA while remaining bound to other nuclear proteins.

In sum, although refinements continue to be made in our understanding of steroid receptor disposition within cells, the basic two-step model proposed in the late 1960s remains valid. To recapitulate, the interaction of the steroid hormone with its cognate receptor leads to an alteration in the physical state of the protein, resulting in its conversion to a form with increased affinity for DNA. Whether the receptor ever resides in the cytosol after its synthesis and whether the receptor ever exists in a soluble form within the nucleus are interesting issues, but ones that are perhaps ancillary to the mechanisms by which these proteins modulate gene activity.

REGULATION OF GENE EXPRESSION

Shortly after the discovery of steroid receptors, several groups postulated that physiological changes induced by steroid (and perhaps other classes of) hormones might be a result of alterations in gene expression [for reviews see (2, 3, 7)]. The then recent discoveries of messenger RNA and the elucidation of gene regulatory mechanisms in bacteria made this suggestion quite fashionable. Development of tools by which to analyze specific proteins in well-defined systems has allowed this prediction to be proven. Suggestive experiments also implied that specific messenger RNAs accumulated in response to treatment of cells with these hormones. Two noteworthy sets of experiments deserve men-

tion. First, Sekeris (19) was ahead of his time in utilizing in vitro translation to document that ecdysone-stimulated increases in *dopa* decarboxylase are due to increased production of the corresponding mRNA. Second, Tomkins and his colleagues utilized metabolic inhibitors in novel ways to suggest that the induction of tyrosine aminotransferase (TAT) by glucocorticoids in HTC, rat hepatoma cells, is due to a direct action of the glucocorticoid-receptor complex on TAT mRNA production (20). Subsequent analyses in a large variety of systems confirmed these general notions. It was not, however, until the discovery of reverse transcriptase and the advent of nucleic acid hybridization and cloning techniques that detailed mechanistic studies on gene activation by steroid hormones became a reality. Since that time, tremendous advances have been made in characterizing the basic principles involved in steroid-mediated induction of gene expression. A general requirement for understanding the detailed mechanisms for gene activation by steroid hormones has been to obtain the DNA encoding the regulated gene product, in particular the region(s) of the DNA involved in the hormonal responsiveness of that gene. The development of recombinant DNA techniques has allowed investigators to obtain unlimited quantities of these important DNA sequences and to test whether, for example, SR complexes interact directly with the DNA adjacent to the coding region. The major focus of this review will be on systems in which the availability of the appropriate DNAs has facilitated a dissection of the molecular aspects of steroid-regulated gene expression.

RECEPTOR STRUCTURE

There is a paucity of data regarding the structure of steroid receptors as it pertains to their role as gene-regulatory molecules. Nevertheless, it is worthwhile at this juncture to briefly review some of the salient features of these proteins and to point out areas that remain to be explored. Until very recently, all data regarding these receptors were derived from their detection with radioactive ligands. The advent of affinity-labeling reagents and monoclonal antibodies for several of these receptors has facilitated the undertaking of more refined structural analyses.

Unactivated Receptors

The unactivated form of steroid receptors has generally been obtained by homogenizing cells at low temperatures in hypotonic buffers. If kept at low temperatures, the receptors remain in this state even when bound to hormone and acquire DNA-binding capabilities only when warmed, exposed to high salt, or subjected to a variety of other experimental conditions [reviewed in (3, 16)]. The recent observation that transition metal oxyanions (molybdate has been used most extensively) stabilize the unactivated form of receptors has

facilitated their characterization (21, 22). Ion-exchange chromatography, isoelectric focusing, and aqueous two-phase partitioning have been used to assess receptor charge. The bulk of the experimental results indicates that the unactivated form of steroid receptors is acidic, with isoelectric points between 4 and 6. Sucrose gradient sedimentation and gel filtration have been the standard means by which to determine the size and shape of steroid receptors. Striking similarities are observed among receptors for various classes of steroids; in all cases studied to date, the unactivated receptor appears to exist as a multimer having a molecular weight of 200–300 Kd, with sedimentation values of 8–10S and Stokes radii of 7–10 nm. Furthermore, the receptors exist as prolate ellipsoids with axial ratios ranging from about 10–20. These and other properties of individual steroid receptors are summarized in recent reviews (16, 23, 24).

Activated Receptors

Most studies of the activated form of steroid receptors have utilized receptors that have been activated in vitro. Various experimental approaches support the notion that receptors lose net negative charge upon activation, resulting in proteins with isoelectric points of 5.5–7.0. Recent evidence indicates that glucocorticoid receptors exist as phosphoproteins in vivo (25), that progesterone receptors can be phosphorylated in vitro and may be phosphoproteins in vivo (26, 27), and that inhibitors of phosphatases such as molybdate and vanadate prevent activation whereas incubation with calf alkaline phosphatase activates receptors (see 24). The speculative hypothesis therefore exists that the activation of steroid receptors involves dephosphorylation of the receptor protein itself.

The activated forms of steroid receptors always have lower sedimentation (S) values than the unactivated forms. Conversion of the 8–10S form to a 3–4S form is characteristic and, since this alteration is associated with conditions that would dissociate oligomeric structures (e.g. high salt, elevated temperature, and dilution), it seems likely that the most stable activated form of steroid receptors is a monomeric structure. Preliminary evidence suggests, however, that a multimeric form may be involved in gene activation (28).

Except for the chick progesterone receptor, which appears to be composed of two dissimilar hormone-binding subunits of about 110 and 80 Kd (29), all the steroid receptors seem to contain a single species of protein ranging in molecular weight from about 60 Kd for the Vitamin D receptor to about 120 Kd for the androgen receptor [reviewed in (16)]. The chick progesterone receptor appears to consist of a small DNA-binding subunit (A) and a large non-DNA-binding subunit (B).

Extremely illuminating experiments relating to receptor structure have recently been performed using radioactively labeled covalent-affinity reagents

for the glucocorticoid, progesterone, estrogen, and androgen receptors. Of particular note is the observation that under denaturing conditions of SDS-polyacrylamide gel electrophoresis, the unactivated and activated forms of the receptor are indistinguishable. Similar conclusions have been reached using monoclonal or polyclonal antibodies to, among others, the glucocorticoid (30, 31), progesterone (32), and Vitamin D (33) receptors. Lastly, purification of glucocorticoid (34) and estrogen (35) receptors in their activated state confirms the generality that, with the exception of the avian progesterone receptor, steroid receptors are composed of single hormone-binding polypeptides.

Functional Receptor Domains

Our knowledge of the functional domains inherent in the monomeric form of steroid receptors comes primarily from partial proteolysis studies of the rat and mouse glucocorticoid receptor. This receptor of 90–95 Kd appears to be composed of three distinct domains that can be resolved by judicious or in some cases accidental use of proteases (23, 26). A fragment of about 40 Kd that retains both hormone and DNA binding activities can be released from the receptor by mild treatment with chymotrypsin and occurs spontaneously in cell extracts (23, 36–38). Further digestion with trypsin-like proteases yields the so-called mero-receptor (23, 39) of approximately 23 Kd that contains only the hormone binding domain. The region of the receptor that has no obvious function contains the major antigenic determinants (36) and is likely to play a crucial role in modulating DNA binding activity, perhaps by serving as a binding domain for other nuclear proteins or by facilitating proper receptor-receptor interactions. The physiological importance of this region is indicated by the behavior of mutant forms of the glucocorticoid receptor that are ostensibly devoid of this "modulating" domain. In mouse S49 lymphoma cells carrying this mutated form of the receptor, the so-called nt^i receptor, the typical cytolytic response to glucocorticoids does not occur (40, 41). Furthermore, the nt^i receptor exhibits increased binding affinity for non-specific DNA sequences (42). Recently, receptor fragments devoid of the "modulating" domain have been prepared in vitro from purified receptor. These fragments retain the ability to recognize and bind to specific DNA sequences adjacent to glucocorticoid inducible genes, albeit with reduced affinity when compared to native glucocorticoid-receptor complexes (28, 43). Thus, the role of this region of the receptor may be to aid in discriminating between specific and non-specific DNA binding sites. This will be discussed in more detail in a following section. Those readers who wish more detail on the characteristics of proteolytic fragments derived from the glucocorticoid receptor and the mutant forms of the receptors, as well as information on other steroid receptors, are directed to one or more of the following reviews (16, 23, 44, 45).

GLUCOCORTICOID INDUCIBLE GENES

Glucocorticoid Induction of MMTV (A Summary of the Viral Life-Cycle)

Viruses have played a leading role in deciphering the intricacies of biological regulatory mechanisms in both procaryotic and eukaryotic organisms. In the case of steroid hormone action, the fortuitous observation that glucocorticoids stimulate production of mouse mammary tumor virus (MMTV) in cultures of mouse mammary carcinoma cells (46) has led to what is now perhaps the best characterized model of how steroids regulate gene transcription. MMTV is a typical retrovirus containing a single-stranded RNA genome that replicates via a DNA intermediate called the provirus. Viral DNA is synthesized by the virion-associated reverse transcriptase and eventually becomes covalently integrated, apparently in a random fashion, into the host cell's chromosomes. At that point, the proviral DNA becomes a stable genetic element akin to any other cellular gene encoding a polypeptide sequence and can be viewed as being equivalent to the genes encoding TAT, globin, or albumin, for example. It is transcribed by RNA polymerase II (47) to yield a primary transcript of about 9,000 bases in length that is utilized both to produce some of the viral proteins and to encapsidate into progeny virions. In addition, smaller spliced RNAs are synthesized as templates for translation of the major viral glycoproteins (gp52 and gp36), as well as a protein of unknown function whose production is restricted to a subset of mammary cells [for recent reviews, see (48, 49)].

In order to appreciate the intricacies of this system, the reader should be aware of the general structure of the MMTV provirus (see Figure 1). As a consequence of the fascinating process by which reverse transcriptase generates the provirus (50, 51), sequences represented within the 3' and 5' ends of viral RNA are duplicated to generate a DNA molecule containing direct repeats at its termini. These so-called long terminal repeat (LTR) sequences contain most, if not all, of the regulatory signals involved in the control of viral gene transcription (50, 51). In the case of MMTV, the LTR is approximately 1,350 base pairs in length, with about 130 of these arising from the 5' end of viral RNA. Thus, it is convenient from the viruses' and the investigator's point of view that the LTR that contains the viral regulatory signals is encoded within the viral RNA itself.

INDUCTION OF MMTV RNA The great interest in studying MMTV as a model system has been predicated on early studies showing that the glucocorticoid-mediated increase in production of virus by mammary tumor cells is a consequence of increased production of viral RNA (52, 53). More importantly, the induction of viral RNA is rapid, independent of ongoing protein synthesis, and

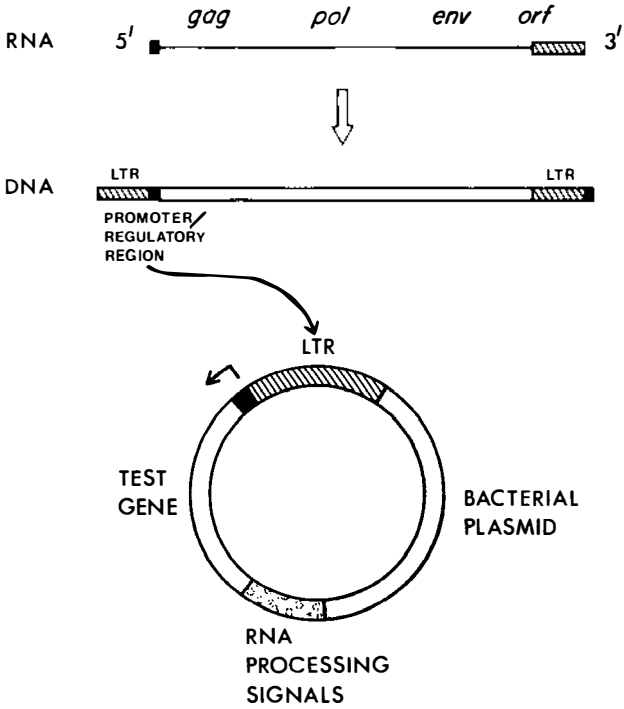


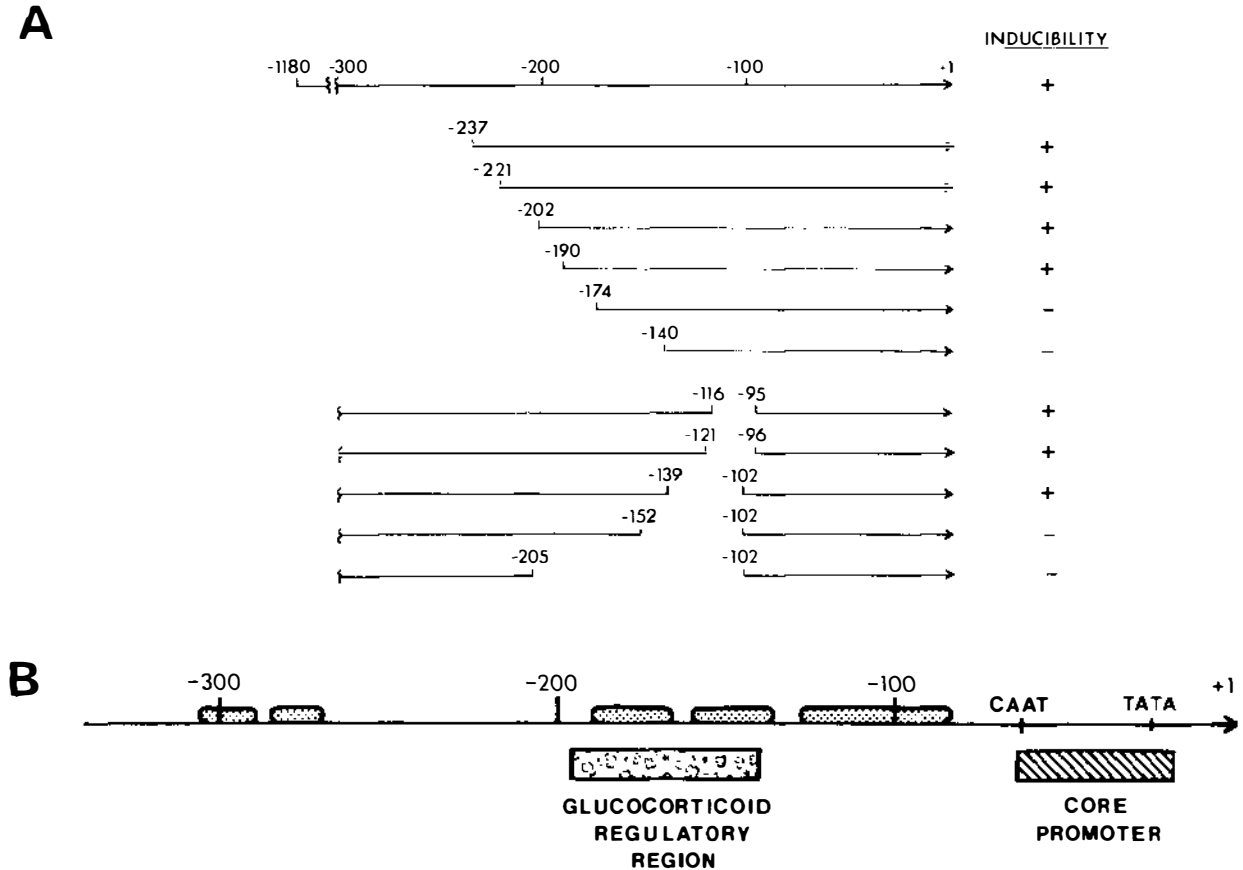
Figure 1 The structure of MMTV RNA, DNA, and a prototypical chimeric gene. The intact viral RNA (approximately 9000 bases) is illustrated with the coding regions indicated: *gag* = group-specific antigens or coat proteins; *pol* = viral polymerase or reverse transcriptase; *env* = envelope glycoproteins; *orf* = a protein of unknown function encoded within a 3' open reading frame. The linear form of double-stranded MMTV DNA contains long terminally repeated (LTR) sequences derived from both the 3' and 5' ends of viral RNA; these are indicated as the solid 5' region (130 base pairs) and the slashed 3' region (approximately 1200 base pairs). This structure is maintained when the viral DNA integrates into chromosomal DNA to form the provirus. A generic structure for chimeric recombinant plasmids is shown in which one of the MMTV LTR sequences (which contains the promoter and regulatory region) is placed adjacent to a marker or test gene whose activity can be easily measured after transfection of the DNA into recipient tissue culture cells. In addition to the LTR promoter/regulatory region and the test gene itself (clear), sequences required for RNA processing usually from the virus SV40, and all or a portion of a bacterial plasmid (stippled) that allows manipulation and propagation of the DNA in *E. coli*, are included in the recombinant molecule. [For a more detailed discussion, see (65).] Similar constructions have been utilized to characterize the promoter and regulatory regions of several steroid-inducible genes.

mediated by the cellular glucocorticoid receptor (54, 55). Particularly noteworthy was the observation that the accumulation of viral RNA is due to an increase in its rate of synthesis (56, 57); this was the first experimental demonstration that steroid hormones could directly affect the transcription of a specific gene. Lastly, infection of heterologous (i.e. non-mammary) cells by MMTV revealed

two crucial points: (*a*) glucocorticoid inducibility is retained in a variety of cell types, including mink lung, cat kidney, and rat liver (58, 59), and (*b*) the sites of proviral integration in host-cell DNA are ostensibly random (60, 61). Thus, the signals that impart glucocorticoid sensitivity to MMTV must be encoded within the viral genome. An additional point borne out by these studies is that the functional region of the glucocorticoid receptor must be highly conserved, since the induction of MMTV RNA is retained in cells from widely divergent species.

TRANSFECTION OF MOLECULARLY CLONED MMTV DNA The advent of molecular cloning has resulted in prodigious advances in our abilities to dissect the coding, non-coding, and regulatory components of specific DNA sequences. The first such experiments with MMTV entailed introduction of cloned proviral DNA into mouse fibroblasts (62, 63) by the newly developed methods of DNA-mediated gene transfer or transfection (64). These studies corroborated the information previously garnered by analysis of virally infected heterologous cells (see above) and strengthened the argument that the proviral DNA itself carries glucocorticoid regulatory sequences. The likely possibility that such sequences reside near the promoter region was tested by assessing the hormone responsiveness of chimeric genes in which an MMTV LTR is fused to heterologous coding sequences (see Figure 1). In all cases, the production of the fused gene product exhibits glucocorticoid inducibility in a variety of tissue-culture cell lines. Among the proteins that have been expressed in a hormone-dependent fashion are mouse dihydrofolate reductase (65), v-*ras* of the Harvey sarcoma virus (66), herpes simplex thymidine kinase (67–69), and the *E. coli* enzymes β-galactosidase and XGPRT (70, 71). These experiments provide convincing evidence that sequences within the MMTV LTR are sufficient to confer hormone sensitivity on the MMTV promoter.

In addition to studies employing chimeric genes, Fasel et al (72) and Yamamoto and colleagues (73) have introduced fragments of the MMTV genome into mouse L cells by co-transfection with the herpes virus TK gene. Again, production of MMTV RNA is hormone inducible. However, a surprising result was obtained by Yamamoto et al (73), who found that glucocorticoids also stimulate the production of MMTV RNA in cells transfected with non-LTR DNA fragments. As will be discussed below, specific binding sites for the glucocorticoid-receptor complex exist not only within the LTR but within internal viral DNA fragments as well (74). It is tempting to speculate that MMTV may harbor the vestiges of multiple glucocorticoid-regulated promoters that were once derived from cellular genes. This may reflect the possibility that, during the rapid evolution of the virus, the acquisition of multiple regulatory regions conferred upon it a selective advantage.



MAPPING THE GLUCOCORTICOID REGULATORY REGION WITHIN THE LTR By studying the behavior of chimeric genes that have had specific portions of the LTR removed, several groups have defined the region of DNA required for hormonal responsiveness (Figure 2A). The MMTV LTR contains a so-called TATA box and a CAAT sequence approximately 25 and 65 nucleotides respectively upstream from the start of transcription (Figure 2B). These short nucleotide sequences are thought to be important signals for the initiation of transcription by RNA polymerase II [for review see (75)]. By convention, nucleotides upstream of the transcription initiation site are denoted by negative numbers; thus, a deletion that removes all but 65 nucleotides upstream of this site is denoted as a −65 deletion.

Majors & Varmus (69) have reported that an LTR deleted to residue −190 retains hormone inducibility, whereas one deleted to residue −140 does not. Similar data from Hynes et al (76) and Buetti & Diggelmann (68) indicate that inducibility is retained in molecules containing about 200 residues upstream of the initiation site, whereas those retaining only 137 nucleotides lose hormone responsiveness. Lee and Ringold and their colleagues (77, 78) have also found that a −221 deletion is glucocorticoid responsive, whereas a −174 deletion is not. Consistent with these observations, Chandler et al (79) and Hager et al (80) have found that hormonal sensitivity is conferred by small regions of the LTR. The sum of these deletion analyses indicate that the 5' border of the regulatory region resides between residues −190 and −174.

Internal and 3' deletions have also been constructed that delimit the extent of the regulatory region. Hynes et al (76) showed that sequences downstream of −52 are not necessary for hormonal response. Majors & Varmus (67) and Lee and colleagues (77, 78) found that deletion of sequences between −98 and

Figure 2 (A) Deletion mapping of the glucocorticoid regulatory region within the MMTV LTR. The top line represents the entire MMTV LTR; the arrow at residue +1 indicates the site at which transcription initiates; the position −100 indicates 100 base pairs upstream of that site. The portion of the LTR remaining in each deletion mutant is indicated by a solid line; the end points of the deletions have been determined by DNA sequence analysis. Inducibility (+) denotes positive stimulation of expression by the glucocorticoids of a test gene fused to the indicated LTR fragment. This figure is a composite of selected deletion mutants analyzed by several groups (68, 69, 76–78). (B) Summary of the functional regions and glucocorticoid-receptor (GR) complex binding sites within the MMTV LTR. The glucocorticoid regulatory region has been identified by analysis of deletion mutants, as shown above. The core promoter is the minimal region required for basal levels of transcription (76, 78, 80) and includes the characteristic TATA and CAAT boxes [see (75)]. Indicated as the stippled, hot-dog shaped regions are the sites protected from DNase digestion by purified GR complexes as reported by Payvar et al (28). The reader should note that the exact GR binding sites are somewhat different according to Scheidereit et al (111). Two major conclusions derived from this evidence are that the functional glucocorticoid regulatory region encompasses strong GR complex binding sites but that not all binding sites are functional as hormone-regulatory sequences.

−120 and between −97 and −138 respectively do not abrogate inducibility, whereas deletion of sequences between −103 and −151 does. Thus, it appears that the 3' border of the regulatory region resides between residues −138 and −151. A deletion that removes residues −103 to −158, however, retains full inducibility (78). The reason for the anomalous behavior of this mutant is not clear, but it is possible that a fortuitous reconstruction of a functional regulatory region has occurred. This is plausible since there appear to be multiple binding sites for the glucocorticoid-receptor complex within this region (see below).

In addition to delineating the sequences important in responsiveness to hormone, these deletion studies have also shown that the regulatory region is distinct and dissociable from the core promoter region (i.e. those sequences required for appropriate initiation by RNA polymerase at levels equivalent to those seen in the absence of hormone). Dobson et al (81) have reported that a −64 deletion retains the ability to produce RNA that initiates at the appropriate site within the LTR. The level of RNA produced (in the presence or absence of hormone) is comparable to that seen in non–hormone treated cells containing DNAs fused to the full-length LTR. Similar conclusions have been reached by Majors & Varmus (67), Hynes et al (76), and Chandler et al (79). In contrast to these results, Hager's and Khoury's laboratories (80, 82) have reported that, upon deletion of the hormone regulatory sequences, the uninduced level of transcription increases to that seen when the intact LTR is hormone stimulated. These researchers suggest, therefore, that the regulatory region functions as a negative control element and that hormone stimulation relieves its negative effect. Such results have not been seen by any other investigators, and it is difficult to ascertain what experimental details may account for these rather disparate results. The presence of a strong promoter-enhancing sequence (see below) in Hager's & Khoury's constructions may be responsible for this effect.

Several groups have recently reported that the glucocorticoid regulatory region can confer hormone inducibility on heterologous promoters. When the region upstream of residue −102 of the MMTV LTR, which lacks the MMTV core promoter, is fused to promoter fragments derived from the herpes virus TK gene (79), the Rous sarcoma virus LTR (69), the SV40 early promoter (83), and the adenovirus major late promoter (84), expression of the linked marker gene becomes glucocorticoid inducible. Thus, there is no obvious requirement for specific interactions between the regulatory region and the core promoter.

A revealing aspect of both the deletion analyses and the characterization of hybrid promoters is that the absolute spacing between the core promoter and the hormone regulatory region need not be constant. Molecules in which the two elements have been brought closer together by deletion of internal sequences function perfectly well, and several of the functional hybrid promoter constructs move the regulatory region further away from the site of transcription initiation. Chandler et al (79) have reported that the hormone regulatory region

can even be placed 1,000 base pairs away and in the opposite orientation from the promoter and still retain at least partial ability to promote glucocorticoid-inducible expression of a linked TK gene. Hynes et al (76) have also reported that if the MMTV LTR is fused to the herpes TK gene with its own promoter intact, glucocorticoid-stimulated transcripts that initiate at each of the promoters can be detected in transfected cells. These observations have led to the proposal that the glucocorticoid regulatory region of MMTV acts as a hormone-dependent enhancer (79). Enhancers are rather remarkable DNA sequences first found in SV40 (85–87) that can activate promoters from hundreds or even thousands of base pairs away in an orientation-independent fashion. The mechanism(s) by which enhancers activate transcription remains obscure; however, since they exhibit both species and tissue specificity, they are most likely to be sites of protein-DNA interactions (88, 89). The fact that the glucocorticoid regulatory region and enhancers have some properties in common suggests that enhancer-binding proteins may share functional characteristics with steroid receptors. Intriguingly, however, the MMTV promoter is responsive to the SV40 enhancer in the presence or absence of glucocorticoids and to the MuLV enhancer in the presence of glucocorticoids (65, 89a); this suggests that the mechanisms by which enhancers and the SR-complex activate transcription are distinct.

Other Glucocorticoid Regulated Genes

Examples of glucocorticoid regulated genes abound in the literature. It is not my intent to chronicle all such examples, but rather to provide unifying themes from the most extensively studied genes. Human and rat growth hormone genes are induced two- to threefold by glucocorticoids after gene transfer into mouse fibroblasts (90, 91). In the case of the rat gene, this reflects a similar induction profile in rat pituitary-cell cultures (92). The sequences responsible for the glucocorticoid effect appear to reside within 500 base pairs of the transcription initiation site (93).

α2u-Globulin is a protein of about 19 Kd that is produced in the liver and secreted in the urine of male rats (94). In the animal, normal levels of this protein require a combination of hormones, including glucocorticoids, androgens, growth hormone, thyroxine, and insulin (95, 96). Androgens will induce production of this protein in females, but only after ovariectomy; thus, estrogens appear to play a dominant inhibitory role (97). In tissue culture, only the glucocorticoid response has been reproduced. Unlike MMTV, however, the induction of α2u RNA is dependent on on-going protein synthesis and therefore is probably not due to a direct activation of the gene by the glucocorticoid receptor complex (98). It is interesting, therefore, that a genomic clone of α2u globulin transfected into mouse L cells retains glucocorticoid sensitivity (99). Perhaps there is a general class of glucocorticoid-inducible proteins in

cells as disparate as hepatocytes and fibroblasts that is capable of mediating the induction of this RNA. Further delineation of the molecular details involved in the complex response of this gene to other hormones awaits the development of appropriate hormone-responsive tissue-culture systems or the introduction of altered forms of the gene into mouse embryos and their subsequent analysis in transgenic mice.

Several other glucocorticoid-responsive genes have recently been cloned and sequences of the 5' flanking regions have been determined. Of particular interest is a short nucleotide sequence shared between the tyrosine aminotransferase (TAT) and tryptophan oxygenase (TO) genes, both of which are glucocorticoid-inducible in rat liver (100). This sequence is related to the consensus hexanucleotide T-G-T-T-C-T that is found in all of the high-affinity glucocorticoid-receptor binding sites in MMTV, hMT-IIa, and chick lysozyme (see below).

METALLOTHIONEIN A cellular gene that has been extensively characterized and that responds to glucocorticoids in a fashion akin to that of MMTV is the human metallothionein-IIa gene (hMT-IIa). Metallothioneins (MT) constitute a family of small proteins that bind heavy metals. Their production can be induced by the heavy metals themselves and in some but not all cases by glucocorticoids (101). The hormonal induction of these genes is a primary effect and is at the level of transcription, as determined by direct measurement of RNA synthesis (102, 103). The mouse MT-I gene, when transfected into L cells, retains cadmium inducibility but not glucocorticoid sensitivity (104). Unlike this gene, the hMT-IIa gene retains both metal and hormone sensitivity when transfected into mouse cells (105). Deletion analysis of chimeric genes carrying the hMT-IIa promoter region fused to a TK coding region has shown that the 5' border of the region of DNA required for glucocorticoid responsiveness lies between residues -268 and -236 (105). As in the case of MMTV, the core promoter can be dissociated from the hormone regulatory region. The hMT-IIa region also appears to have the capacity for promoting transcription when placed at a distance and either 3' or 5' to the promoter (M. Karin, personal communication). Thus, again, the glucocorticoid regulatory region may be acting in a fashion analogous to viral enhancer sequences. The MT genes, as mentioned above, also respond to metals; analysis of the deletion mutants clearly indicates that the sequences involved in this response are distinct from those involved in hormonal induction (105).

LYSOZYME The chick lysozyme gene can be induced by estrogens, progestins, androgens, and glucocorticoids each via its own receptor in the chick oviduct (106). The induction of ovalbumin in this system will be discussed below. Renkawitz et al (107) have recently constructed a chimeric gene in

which the lysozyme promoter region is fused to the coding region for the SV 40 T antigen. No glucocorticoid or estrogen response could be detected by immunofluorescence assay for T-antigen after transfection of this DNA into HeLa or MCF-7 cells. These are both human cell lines that contain functional glucocorticoid receptors; MCF-7 also contains estrogen receptors. However, when the DNA is microinjected into chick oviduct cells but not into fibroblasts, the fusion gene retains responsiveness to glucocorticoids and progesterone (108). Analysis of 5' deletion mutants indicates that the 5' border of the regulatory region for both classes of steroids lies between residues −208 and −164 (108). Further fine mapping will be required to test whether exactly the same sequences are necessary for responsiveness to both progesterone and glucocorticoids.

GLUCOCORTICOID RECEPTOR BINDING TO SPECIFIC DNA SEQUENCES

As suggested by the observation that steroid receptors are DNA binding proteins (see above) and by analogy with known prokaryotic gene-regulatory proteins, the hypothesis has been put forth that stimulation of transcription requires high-affinity interactions with specific DNA sequences [reviewed in (3)]. The selective binding of the glucocorticoid-receptor (GR) complex to fragments of MMTV DNA was first reported by Payvar et al (74). Using a nitrocellulose filter binding assay, they found that DNA fragments derived from internal regions of MMTV are recognized preferentially by highly purified GR complexes compared to bacterial plasmid sequences. The significance of GR binding sites within the structural portions of the MMTV genome remains unclear; however, as mentioned earlier, vestiges of glucocorticoid-regulated promoters may exist within these regions of the viral genome. Although these first studies detailed what may be an unusual situation, subsequent efforts in several laboratories have focused on GR binding to LTR sequences. Both by filter binding assay and by direct visualization in the electron microscope, it has become clear that the LTR harbors multiple specific binding sites for the GR complex (28, 74, 109, 110). All of the binding sites reside upstream of the core promoter. By employing monoclonal antibodies to the GR, Schneidereit et al (111) have convincingly demonstrated that the binding is indeed due to the receptor protein itself. Using a DNA competition assay in which crude receptor preparations are used for detecting DNA binding sites, Pfahl has also found that sequences upstream of the promoter are efficient sites for GR binding (112).

More refined binding studies have been reported in which purified GR complexes are used to protect LTR fragments from degradation with DNAse I.

The results of these experiments, summarized in Figure 2B, indicate that there are several binding domains within the LTR; these are located between residues −305 and −289, −283 and −269, −189 and −166, −159 and −135, and −127 and −84, according to Payvar et al (28), and between residues −192 and −164 and −127 and −72, according to Schneidereit et al (111). The two domains furthest upstream appear to contain lower affinity sites, as determined by nitrocellulose and electron microscopic binding assays, whereas the highest affinity domains appear to be the ones between −189 and −135 (28). This is particularly gratifying, since this is exactly the region that functions as the gene regulatory region of the LTR as determined by the deletion analyses described earlier. It is noteworthy that each of the binding domains appears to be capable of binding GR complexes independently and that no evidence for cooperative interactions has been reported. As alluded to earlier, the binding form of the receptor, when viewed in the electron microscope, appears to exist as an oligomer of two or perhaps four subunits. Finally, alteration of one domain (i.e. by insertion of a short DNA fragment) affects only that binding site; in agreement with the deletion studies, the only such DNA insertions that affect inducibility lie within the −189 to −135 regions (113). It is interesting to note that the two most upstream regions between −305 and −269 and the large downstream region between −127 and −84 appear to be neither required nor sufficient to support hormone-stimulated transcription from the MMTV promoter. Deletions and alterations of these regions do not affect inducibility, in contrast to the sequences between −189 and −135, which appear to be highly susceptible to such alterations.

Nitrocellulose filter binding and DNAse I footprinting of partially purified GR complexes to the hMT-II promoter region have recently been reported (114). The results are consistent with those found for MMTV and are not confused by the presence of several ostensibly irrelevant binding regions. A single region that lies between residues −266 and −241 is protected from digestion with DNAse I by the GR complex. As summarized earlier, this is consistent with data indicating that the hormone regulatory region resides downstream of residue −268 (105).

In experiments virtually identical to those just described, the GR complex from rat liver has been found to bind specifically to sequences upstream of the chick lysozyme transcription initiation site (108). A strong binding site resides between nucleotides −74 and −39, whereas a weaker site exists between residues −208 and −161. The latter binding region coincides almost precisely with the sequences required for a functional glucocorticoid (and progesterone) response in chick oviduct cells (108). The specific binding of the rat liver GR complex to a chicken gene provides another example of the conserved function of this steroid receptor.

A Common DNA Sequence in the GR Binding Domains

The sequence T-G-T-T-C-T or derivatives of the degenerate octanucleotide $^{T}_{A}CTG^{T}_{A}TCT$ are present, sometimes in multiple copies, within all of the binding regions to which the GR complex binds in vitro (28, 111, 114). These include all of the binding regions in the MMTV LTR, the internal MMTV fragments, the hMT-IIa promoter, and the chick lysozyme promoter. In addition, this same hexanucleotide is present at approximately residue −100 in the promoter regions of TAT and TO, two rat liver genes that are glucocorticoid inducible (100).

That this hexanucleotide is important for GR receptor binding is corroborated by experiments in which the contacts between the receptor and MMTV DNA have been studied (115). In these studies, MMTV LTR sequences were subjected to methylation at the N−7 position of the G residues within the hexanucleotide. When methylated, the GR complex is unable to bind to that specific domain but not the others. Furthermore, all of the G residues within the hexanucleotide in four separate binding domains are protected from methylation by dimethyl sulfate when the GR complex is prebound. Similar data have been obtained in studies of GR binding to the hMT-IIa regulatory region (114).

It is important to point out that in both the MMTV LTR and the chick lysozyme promoter, not all bona fide binding sites are functional as hormone regulatory regions, at least as tested by transfection analysis. As an example, even though the binding sites between residues −127 and −84 are preserved in various deletion mutants of the LTR, such molecules are incapable of supporting a glucocorticoid response (69, 77, 78). What then are the crucial elements that constitute a functional regulatory region? Although there is no answer to this question at present, the methylation protection experiments of Schneidereit & Beato may provide a clue (115). They have noted that the protected G residues in the MMTV regulatory region are 10 base pairs (one turn of the helix in B-form DNA) apart and are therefore on the same face of the helix. The N−7 position of the G residue, which is the one susceptible to methylation, is in the major groove of the double helix. Inspection of the sequence in this region shows clearly that there are two pairs of G residues that could contact the receptor within the major groove between nucleotides −170 and −189. Thus, a dimer of GR complexes could bind to this region of DNA. In the case of the non-functional sites this paired arrangement of contacts does not exist, and thus dimer formation may not occur. I point out, however, that the available evidence from electron microscopy indicates that tetramers of GR complexes bind to all sites (28). Thus, it may be another aspect of the symmetry of the paired G residues (or more likely some other characteristic of the surrounding DNA sequence) that is important in allowing for a productive interaction of

receptor. More detailed studies of the functional regions will be required to gain further insight into this problem.

Affinity of the Receptor for DNA Sequences: Is That All There Is?

Does the binding of GR complexes to naked DNA in vitro reflect the situation within a cell? Is the binding affinity of the GR complex for specific sequences relative to extraneous DNA sufficient to account for its ability to find limited number of sites in vivo? Although there have been no binding constants reported, the relative affinities between specific and non-specific sites appear to be several thousand–fold (112). These values seem to be insufficient to account for the high degree of selectivity inherent in steroid activation of genes; researchers have estimated that a minimum 10^5–10^6 fold difference in binding affinity between specific and non-specific sequences might be required for activation of a gene by steroid receptors (116). Thus, additional factors probably are required for biologically relevant binding to occur in vivo. Along these lines, Payvar et al (117) have recently identified a 72 Kd protein that copurifies with the GR complex; this protein is itself incapable of binding to DNA but increases the affinity of the GR complex for LTR sequences. Additional proteins, both chromosomal and non-chromosomal, are likely to be involved in the formation of a functional GR complex at specific sites. In addition, one must remember that other proteins associated with the chromatin may mask non-functional binding sites. In such a case, a feature of the authentic regulatory regions with which GR complexes interact might be that they are in an open configuration relative to the surrounding chromatin. Speculation on events subsequent to the binding interactions will be presented in a following section.

SEX STEROID REGULATION OF GENE EXPRESSION

The Chick Oviduct

The production of egg white proteins in the chick oviduct is dependent on its exposure to estrogen and progesterone (7). The tubular gland cells of the immature chick oviduct are stimulated to proliferate and differentiate by exogenous administration of estrogen; during this time, ovalbumin, conalbumin, ovomucoid, and lysozyme become major products of these cells. If the hormone is withdrawn, egg white protein synthesis decreases but can be restimulated by a second injection of estrogen. In a primed oviduct, this so-called secondary induction occurs more rapidly and is generally of greater magnitude than in the primary induction. Moreover, in a primed oviduct, not only estrogen but progesterone, glucocorticoids, and androgens are able to

induce the production of several of the egg white proteins (106). Each of these hormones appears to act through its cognate receptor.

Nucleic acid hybridization and cloning technologies were first utilized in the steroid hormone field to assess the effects of estrogen and progesterone on ovalbumin RNA levels (118). The pioneering work of O'Malley and Schimke and their colleagues clearly documented that the hormones increase mRNA levels (119, 120). Although estrogen does have an effect on the turnover rate of ovalbumin mRNA (121), the major impact of the hormones is clearly at the level of transcription (122, 123). Extensive and excellent reviews of the early characterization of this system are available (124, 125).

THE OVALBUMIN GENE The ovalbumin gene is one of three tandemly linked related genes (the others are the X and Y genes) that are induced in response to estrogen (125–127). Unlike the ovalbumin gene that responds to estrogen, progesterone, and glucocorticoids in a primed oviduct, the Y gene responds only to progesterone and estrogen, whereas the X gene responds solely to estrogen (126, 127). Furthermore, in a mature laying hen, the X and Y genes are expressed at levels approximately 10% and 1% that of ovalbumin. By far the most extensive characterization has been performed on the ovalbumin gene itself.

THE HORMONE CONTROL REGION(S) OF THE OVALBUMIN GENE Chimeric genes containing the 5' flanking region of the ovalbumin gene fused to the coding region for chick β-globin have been constructed. When globin is introduced into most mammalian cell lines, its expression is directed by the ovalbumin promoter but is not hormone responsive (128). However, in chick oviduct cells the ovalglobin fusion retains estrogen inducibility (129).

More extensive analysis of the ovalbumin regulatory region has been performed by transfection of primary tubular gland cells derived from chick oviduct (129). Analysis of deletion mutants of the ovalbumin 5' flanking sequences indicates that: (*a*) the core promoter lies downstream of residue −95, and basal transcription is heavily dependent on sequences between residues −77 and −48 (128) as well as the TATA box (centered at residue −32), which is generally required for appropriate positioning of the transcription initiation site (75); (*b*) sequences required for the progesterone response are present between nucleotides −222 and −95 (129); the precise boundaries of the functional region have not been identified. As in the case of the glucocorticoid regulated genes already described, the core promoter and the hormone regulatory regions of the ovalbumin gene appear to be distinct and separable. Particularly intriguing are recent results indicating that the responsiveness of this gene to estrogen and glucocorticoids also requires sequences

within the same −222 to −95 region (130). More precise mapping studies will have to be performed to ascertain whether the sequences responsible for each of these hormonal responses are clustered, overlapping, or perhaps even identical.

THE LYSOZYME GENE The chick lysozyme gene is responsive to both progesterone and glucocorticoids. As described above, analysis of deletion mutants in microinjected chick oviduct cells indicates that the left-hand border of the regulatory region lies between residues −208 and −164 for both progesterone and glucocorticoids (107, 108). As in the other steroid-inducible genes, the core promoter and the hormone regulatory regions appear not to overlap.

Mammalian Genes Responsive to Sex Steroids

UTEROGLOBIN The best characterized mammalian gene that responds to progesterone is that for uteroglobin, a protein secreted into the lumen of the uterus that itself has progesterone binding activity. Nucleic acid hybridization studies have documented that its induction in rabbit uteri is consequent to an increase in mRNA levels (131) and that this is due to an increase in the rate of uteroglobin RNA synthesis (132). Interestingly, estrogen is also capable of inducing mRNA levels, although not to the same degree as progesterone; when added together, the hormones appear to have an additive effect (131). Transfection studies with this gene have not yet been reported; however, binding studies with progesterone receptor indicate the presence of specific binding sites in the 5' flanking region (see below).

ANDROGEN-INDUCIBLE PROSTATIC C3 GENE Prostatic steroid binding protein is a tetramer composed of three different polypeptides (C1, C2, C3) whose expression is under strict androgen control (133). The androgen induction of the three is due to alterations in the levels of the corresponding mRNAs and is accomplished at least in part by increased transcription of the genes. Transfection of a C3 genomic clone containing approximately 3.5Kb of 5' flanking DNA into the androgen-responsive S115 mammary carcinoma line results in androgen inducibility of C3 RNA sequences (134).

ESTROGEN-INDUCIBLE GENE PS2 FROM HUMAN MCF-7 CELLS The human breast cancer cell line MCF-7 is estrogen dependent for growth, expresses several estrogen-inducible genes, and undergoes hormone-dependent morphological alterations. Recently, a cDNA encoding a specific estrogen-inducible mRNA (pS2) has been cloned and utilized to document that the induction by estrogens is primary (135). The experiments performed to date are analogous to those described earlier for the glucocorticoid-mediated induc-

tion of MMTV (54) and for the estrogen-mediated induction of vitellogenin in *Xenopus* oocytes (136).

Binding of Sex Steroid Receptors to Specific DNA Sequences

Initial observations that the chick oviduct progesterone receptor could bind to specific sites within the 5' flanking region of egg white genes utilized a DNA competition assay in which the receptor itself does not need to be purified. The results of such experiments indicate that fragments of DNA from the ovalbumin, X, Y, ovomucoid, and conalbumin genes contain high affinity sites for the progesterone receptor. More detailed mapping studies point to a region between residues −250 and −300 of the ovalbumin gene that contains a particularly efficient binding site (137). It is noteworthy that, based on functional studies described above, the region between −250 and −300 is dispensable for progesterone action in vivo (129). Thus, detection of receptor binding sites using a DNA competition assay by itself has not been useful for identifying authentic regulatory regions of the ovalbumin gene.

Nitrocellulose filter binding assays and nuclease protection experiments with chick progesterone receptor have also been reported (138, 139). In these studies, a highly purified A subunit of the chick oviduct receptor has been employed; note that the B subunit, which also binds progesterone, is not a DNA binding protein. The results indicate that the A subunit of the receptor exhibits preferential binding to 5' flanking regions of the ovalbumin and Y genes by a factor of 10 over other DNA sequences. A major binding site in the ovalbumin gene lies between residue −247 and −135, consistent with the region ostensibly required for progesterone responsiveness (137). It is important to recognize, however, that the ten-fold higher affinity of the A subunit for specific sites is probably too low to account for the high degree of selectivity observed in vivo. Thus, additional factors may be involved in the sequence-specific binding of the progesterone receptor to regulatory sites.

The mammalian, unlike the chick, progesterone receptor appears to consist of a single polypeptide chain of about 110 Kd (32). Such a receptor, purified to approximately 50% homogeneity, exhibits binding specificity toward the 5' flanking region of the rabbit uteroglobin gene (140). Two sites that appear to have higher affinities than others reside within nucleotides −394 to −251 and −193 to +10. The functional regulatory region associated with this gene remains to be determined.

Lastly, specific binding of the estrogen-receptor complex to a 5' flanking region of the chicken vitellogenin gene has been reported (141). Both DNA competition and DNAse protection assays indicate a prominent binding region between residues −550 and −660; multiple binding sites are present within this region. Of particular interest in this case, the estrogen receptor binding region corresponds to the location of a DNAse hypersensitive site that appears after

estrogen treatment (142). Whether this region plays any role in estrogen inducibility per se remains to be determined; however, no functional regulatory region has yet been identified more than 250 nucleotides upstream of the transcription initiation site in any of the other steroid-inducible genes characterized to date. Along these lines, it is worth pointing out that a striking sequence homology has been identified around position −140 in five estrogen-inducible genes of the chick (108); the derived consensus sequence ($AAA^{T}_{A}TGG^{A}_{G}C$) bears little resemblance to the binding site identified in the vitellogenin gene; however, its role in estrogen inducibility is only speculative.

HOW DO STEROID-RECEPTOR COMPLEXES STIMULATE TRANSCRIPTION?

Prodigious advances have been made in our understanding of the mechanisms by which steroids regulate gene transcription. Moreover, the emerging data now allow us to focus our attention on a more precise definition of the molecular details involved. As a framework, I present the following possibilities as general mechanisms by which binding of the steroid-receptor (SR) complex to a regulatory region could facilitate increased transcription initiation.

1. SR complexes could relieve a negative effect of the regulatory region on promoter efficiency, perhaps by removing a repressor protein.
2. Direct protein-protein interactions between SR complexes and RNA polymerase may stimulate polymerase function.
3. Local unwinding of the DNA near the RNA polymerase binding site might increase the efficiency of polymerase binding to the core promoter.
4. SR complexes may alter the conformation of the DNA at the regulatory region in such a way as to facilitate the entry of transcription factors that can slide up or down the DNA (even over long distances) in search of poised polymerase molecules.
5. Binding of the SR complex could induce a change in the structure of the chromatin (i.e. by removing nuclear proteins) that could be propagated to the core promoter in such a way as to facilitate polymerase binding; such an alteration could occur over short or long distances.

It is of course difficult at this point to unequivocally discard or accept any of these possibilities. Nevertheless, some may be more likely than others. Since with only one exception, noted above, removing the hormone regulatory sequences from a core promoter does not lead to hormone-independent high-level expression, it seems highly unlikely that the function of the SR complex is to relieve a negative influence of that region on transcription.

Several studies have documented that the glucocorticoid regulatory region of

MMTV functions at various positions relative to the promoter (65, 69, 76, 79). Most of the changes in spacing have been over only 100–200 base pairs; however, in at least one case, partial function was retained even when the regulatory region was placed 1000 base pairs from the promoter. In addition, induction of a distal promoter is seen in some situations despite the presence of a functional intervening promoter. In view of these results, it is difficult to envision how direct interactions of the SR complex with polymerase could be of central importance in the facilitation of transcription initiation. Likewise, local unwinding of the DNA (i.e. over 1–3 turns of the helix), which is of importance in forming stable RNA polymerase complexes in *E. coli,* appears to be of little importance if the regulatory region can be moved over several hundred base pairs. A caveat to these arguments, of course, is the fact that we know little about the organization of these sequences in three-dimensional space. It is possible, therefore, that even when the regulatory region is moved relative to the promoter, additional DNA binding proteins help keep these regions in close apposition. The suggestion that the receptors themselves may be fixed on the nuclear matrix at all times (17, 18) could facilitate such interactions.

Bacterial Models

Positive transcriptional regulators in prokaryotic systems share several characteristics with steroid receptors. Foremost among these, they bind to specific DNA sequences near the corresponding promoters and in some cases (e.g. the cyclic AMP binding protein of *E. coli,* CRP) require a ligand to convert them to a DNA binding form. Whether the mechanisms by which steroid receptors and bacterial protein activate transcription are similar or not remains to be determined.

In the case of the phage λ repressor, which also acts as a positive regulator at the Prm promoter, it appears that transcription is augmented by altering the isomerization rate at which functional (i.e. open) RNA polymerase complexes (143, 144) are formed rather than by altering the affinity of the RNA polymerase itself for the promoter (145). Ptashne and his colleagues have suggested that this stimulation occurs as a consequence of direct protein-protein interactions with the RNA polymerase (146). The data supporting this view are highly circumstantial; however, mutants that bind to the appropriate DNA sequence yet do not stimulate transcription support this hypothesis (145, 147).

Several *E. coli* promoters that are activated by cAMP have been intensively studied [for an excellent review see (148)]. In both the *lac* and *gal* promoters, the cAMP-CRP complex appears to stimulate transcription in two ways: (*a*) by shifting the RNA polymerase binding site by 20 and 5 nucleotides respectively, and (*b*) by increasing RNA polymerase open complex formation at the downstream site (149). Augmented transcription initiation may be due to increasing

the affinity of the RNA polymerase for promoter sites rather than to affecting the isomerization of polymerase from the closed to the open (active) state. Although binding of the cAMP-CRP complex alters the structure of the DNA to form a more compact molecule, evidence indicates that this change is not due to a transition from right-handed to left-handed DNA or from local unwinding of the DNA (148). Indirect evidence suggests that stimulation of transcription by cAMP-CRP may instead involve direct interactions with RNA polymerase (148–151). In the presence of cAMP, CRP appears to stabilize RNA polymerase binding to the upstream promoter, and the affinity of the cAMP-CRP complex for its binding site in the *lac* and *gal* promoters is increased by the addition of RNA polymerase (148–151). However, the distance between the CRP binding site and the start of transcription varies substantially among cAMP-regulated genes, ranging from 35 to 135 base pairs. Thus, one's notions concerning the nature of the putative interaction between the polymerase and CRP must take this into account.

Effects of Steroid Hormones on Chromatin Structure

Transcriptionally active genes in eukaryotes exhibit altered chromatin configurations as detected by their relative sensitivity to nucleases (152). The DNA within relatively large domains encompassing a gene or gene cluster are preferentially digested by DNAse I if the gene has been or is actively being transcribed. Within these domains, specific sites are more sensitive to digestion by 1–2 orders of magnitude (153). These so-called hypersensitive sites (HS) can generally be found within the 5' flanking region of active genes, often at the 3' end of the gene as well, and at times at internal sites in genes (153, 154). Interesting correlations suggest that the appearance of the HS sites is related to transcriptional activation. However, the basis for the appearance of HS sites is not at all clear, and to date no cause-effect relationship has been established between their appearance and the activation of transcription. With this as background, it is intriguing to note that steroid hormones can alter both the general pattern of DNAse sensitivity and HS sites in regulated genes.

GENERAL ALTERATIONS IN DNAse SENSITIVITY Vitellogenin, the precursor of the major yolk proteins, is induced by estradiol in the livers of oviparous vertebrates (155). Four closely related genes encode vitellogenins in *Xenopus laevis,* and recent data from Gerber-Huber et al (156) have shown that at least two of these become generally more sensitive to DNAse in the livers of estrogen-treated males. Similarly, the chick ovalbumin gene is associated with a relatively DNAse-sensitive region of the chromatin that persists even after withdrawal of estrogen (157). Interestingly, the chromatin domain in which the ovalbumin, as well as X and Y genes, resides extends over approximately 100 kilobases and is flanked at the borders of the DNAse-sensitive region by a short middle repetitive sequence, CR1 (158). Thus, these genes probably are fixed in

an open chromatin configuration during exposure of the tissue to hormone. This structure is maintained even after removal of the hormone when the gene is no longer active. This can be viewed as equivalent to a developmental alteration that is required but is itself insufficient for gene transcription. Similar long-lived and perhaps irreversible changes in chromatin structure surrounding the MMTV LTR are seen after glucocorticoid treatment (159).

ACQUISITION OF HYPERSENSITIVE SITES Burch & Weintraub (142) have recently reported that there are three classes of HS sites detectable in the chick vitellogenin gene. Prior to estradiol-mediated activation of vitellogenin expression, HS sites exist within and 3' to the gene in liver and oviduct but not in erythrocytes, brain, or fibroblasts. These sites exist in embryonic liver even prior to development of estrogen receptors. Thus, this class of HS sites (HS-A) may reflect the potential for tissue-specific expression in an estrogen-responsive cell; note, however, that vitellogenin is not expressed in oviduct. A second class of sites (HS-B) are detected in the 5' flanking region of the gene in liver but not in the oviduct after estrogen treatment. These sites remain hypersensitive after removal of hormone and may therefore be involved in the memory phenomenon (i.e. the rapid secondary induction after hormone withdrawal) associated with estrogen induction of vitellogenin in liver (160) and several of the egg-white genes in the oviduct. Lastly, one HS site (HS-C) appears in response to estrogen approximately 700 base pairs upstream of the start of transcription. This site disappears after removal of the hormone and reappears upon re-induction. Such a site is likely to reflect directly an alteration in DNA structure associated with binding of the estrogen-receptor complex. Whether this site is part of the binding site itself remains to be determined. A similar analysis of HS sites in the MMTV LTR reveals the presence of a hormone-dependent and reversible site that indeed maps within the functional glucocorticoid regulatory region (159). It is noteworthy that the HS site seems to coincide with the binding region that is functional in stimulating transcription and not with all receptor binding domains. Mapping of HS sites in the rat TAT gene has revealed the presence of a glucocorticoid-inducible site approximately 2000 base pairs upstream of the start of transcription (161). Again, whether this reflects the presence of a receptor binding site or is the consequence of receptor binding at a distal site is as yet unknown. DNAse HS sites are also present in at least three locations in the 5' flanking region of the actively transcribed ovalbumin gene (162).

Chromatin Structure, DNA Supercoiling, and Transcriptional Activation

Are the observed steroid-induced alterations in chromatin structure responsible for increased transcriptional activity and if so how? Alternatively, are these changes a consequence of the process of gene activation by steroid-receptor

complexes? Although there are no clear answers to these questions at the moment, there are interesting parallels to be drawn with other gene regulatory systems and with proteins that alter higher order structures of DNA.

Several lines of evidence point to the fact that supercoiling of DNA can profoundly affect gene expression [for review, see (163)]. Elegant genetic experiments have implicated the *E. coli* supX gene in the control of transcription, in some cases from promoters that can also be regulated by the cAMP-CRP complex (164). The supX gene encodes the enzyme topoisomerase I (165), one of a class of enzymes that change the degree of DNA supercoiling [for review, see (166)]. Promoters such as the *lac* promoter appear to be active only when the DNA is highly negatively supercoiled by the enzyme DNA gyrase; topoisomerase I relaxes negative supercoils and thus renders the promoter less active. Thus, mutations in supX, which inactivate topoisomerase I, would result in a higher degree of negative supercoiling and effect more efficient transcription from the promoter. Support for this idea comes from the observation that inhibitors of DNA gyrase such as nalidixic acid strongly inhibit the transcription from some promoters but not others (167, 168). The mechanism by which negative supercoiling stimulates transcription is unclear, but perhaps an alteration in the structure of the promoter region facilitates RNA polymerase binding or formation of open complexes (169). These, as we've already discussed, are the functions attributed to the λ repressor and the cAMP-CRP complex. Physical studies of naked DNA have also demonstrated that changes in superhelical density have profound effects on sequences that have the potential for existing in alternative secondary structures [for example, see (170)].

A remarkable example in eukaryotic cells in which supercoiling appears to have a central role in gene expression is that of the yeast mating type genes, which are transcribed from one chromosomal location but not another (171, 172). Using autonomously replicating plasmids in different strains of yeast, Nasmyth (173) has shown that the degree of supercoiling of the DNA seems to determine whether the mating type gene is expressed or not. The product of an unlinked gene, called SIR or MAR, appears to be responsible for controlling the structure of the DNA and may be analogous to the bacterial gyrase. It is noteworthy that the alterations of the mating type genes affect the chromatin structure surrounding the gene profoundly such that nucleosome (histone octamer) phasing may also be affected.

It is clearly premature to do so; nevertheless, it is tempting to speculate that steroid-receptor complexes may also alter DNA and chromatin structure by introducing negative supercoils into DNA. Since in chromatin negative supercoils are taken up by the nucleosome, such alterations may result in dramatic effects on the structure of the surrounding chromatin. As suggested by Yamamoto & Alberts (3), such an alteration may propagate over patches of chroma-

tin, making the region more accessible to transcription factors or polymerase itself. Furthermore, the acquisition of DNase HS sites in the presence of hormone may reflect exposure of a specific DNA sequence that can serve as the recognition site for transcription factors. In each of these cases, the effect of the steroid-receptor complex could be propagated over quite large distances to effect alterations in promoter utilization. Evidence from several deletion and insertion mutants in the MMTV LTR indeed suggest that some property of activating the regulatory region must be propagated to the core promoter (69, 78). The effect could be manifested either by direct propagation of a physical alteration or by the movement of a transcriptional factor in search of a poised transcription complex. This latter hypothesis has also been put forth to explain how viral enhancer sequences can activate promoters from a distance (86).

A final speculation regarding the steroid-receptor complex is the possibility that specific interaction with another protein or set of proteins is responsible for gene activation. One can envision a scenario in which binding of the steroid-receptor complex to the regulatory region serves as the focus for the interaction of a gyrase-like protein that itself is responsible for altering the topology of the chromatin. In such a case, it might be that all steroid-receptor complexes interact with the same or a closely related protein.

DEVELOPMENTAL AND POST-TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION

Steroid hormones have profound effects on cellular physiology, many of which are due to the direct activation of transcription by steroid-receptor complexes. However, the products of the genes activated by a given hormone may in themselves have dramatic effects on gene expression. The notion of such a cascade phenomenon is exemplified by the activation of genes in Drosophila polytene chromosomes by the moulting hormone ecdysone. Ashburner and colleagues (174) identified five gene loci (i.e. the early genes) activated in Drosophila salivary glands in the absence of protein synthesis. In a temporally defined order and incumbent on de novo protein synthesis, a large series of middle and late genes subsequently become activated. Coordinate with the activation of these genes, the early genes return to a quiescent state. This highly regulated developmental program is initiated by a single administration of ecdysone and, although this hypothesis is not yet proven, suggests that the products of the early genes (i.e. those directly activated by the hormone) themselves have profound gene regulatory activities. Also related to such a cascade are instances in which one steroid induces the production of the receptor for a different steroid. The best documented example is the estrogen-mediated induction of progesterone receptors in various tissues [for example, see (175)].

Another case in which a steroid hormone appears to regulate gene expression indirectly is the glucocorticoid-mediated induction of the acute phase reactant, α-1 acid glycoprotein (AGP). The RNA encoding this protein is induced several hundred–fold both in rat liver and in HTC cells, and at least in the latter case it requires ongoing protein synthesis (176–178). Most surprisingly, the induction of the RNA appears not to be at the level of gene transcription (179). One interpretation of these results is that the glucocorticoid-receptor complex directly activates a gene whose product is required for the production of stable AGP transcripts. This novel mechanism of gene regulation may be more general than previously believed; a thyroid hormone–inducible gene in rat liver also appears to be induced by a nontranscriptional mechanism (H. Towle, personal communication). Additional mechanisms may be involved in the ability of steroid (and other) hormones to alter the half-lives of existing mRNAs. For example, the induction of ovalbumin mRNA by estrogen in the chick oviduct is in part due to a stabilization of the RNA (121).

Glucocorticoids have also been implicated in inducing a glycoprotein-processing pathway for MMTV proteins in rat hepatoma cells that could in principle dramatically alter the pattern of cellular membrane and/or secreted proteins (180). Again, one presumes that the hormone-receptor complex activates a gene that encodes in this case a protein-processing factor.

Lastly, steroid hormones have dramatic effects on cell differentiation as exemplified by the estrogen-induced proliferation of tubular gland cells of the chick oviduct (119, 120), as well as the glucocorticoid-mediated conversion of myeloid precursors to macrophages (181) or preadipocytes to adipocytes (182). Similarly, sex steroids are often required for the proliferation and differentiation of tissues involved in the reproduction or maintenance of secondary sex characteristics. In these cases that require complex activation of a large set of coordinately regulated genes, it is indeed likely that products of the steroid-inducible genes have diverse functions in controlling the expression of the tissue-specific genes. Thus, an integrated physiological response to a steroid hormone will entail not only the primary activation of a few specific genes (as a consequence of receptor binding to regulatory DNA sequences) but the concerted interplay of other cellular regulatory factors that affect gene expression at both transcriptional and post-transcriptional levels.

QUESTIONS AND PROSPECTS FOR THE FUTURE

Many of the most pressing questions regarding steroid hormone action have been discussed throughout the text. Nevertheless, it may be useful to restate these more explicitly and to point out experimental approaches and obstacles that may arise.

1. What constitutes an SR complex binding site and what distinguishes a functional binding site from the others? These issues are central to understand-

ing the high degree of selectivity inherent in the cellular response to a particular hormone. For the moment, the most direct experimental approach to this problem will be to undertake fine mapping analysis of binding sites and functional regulatory regions using the powerful techniques of point mutagenesis and transfection. The possibility that two different steroid receptors may recognize the same DNA sequence can also be addressed in this fashion.

2. How does binding of an SR complex stimulate transcription? This, of course, is the central question not only for steroid hormones but for positive regulatory proteins in all organisms. It is sobering that the molecular details of how phage proteins or cAMP-CRP complexes activate transcription in *E. coli* remain hazy. Experimentally, it would be extremely useful if an in vitro, hormone-dependent transcription system could be developed. This will require both luck and a much better understanding of the requirements for polymerase II transcription of genes. Although limited success has recently been obtained in isolating a promoter-specific factor [for example see (183)], no one has yet reported an effect of SR complexes on transcription in a cell-free system. The physical state of the template, in particular with regard to its chromatin structure, may be a crucial aspect of this problem.

3. What is the role of chromatin structure in the control of transcription and how do SR complexes influence it? Although it is clear that chromosomal position profoundly influences the ability of a hormone-responsive gene to be transcribed (60, 184), isolating defined fragments of DNA that are packaged in a native chromatin state is an extremely difficult proposition. Our present understanding of the proteins involved in high-order chromatin structure is limited. Nevertheless, the utility of nucleases and chemical probes in detecting alterations in chromatin structure has been documented. Novel attempts to isolate minichromosomes in bovine papilloma virus vectors containing the glucocorticoid regulatory region of MMTV are underway (185) and have already provided the basis for future attempts to study this most difficult area. These minichromosomes may indeed be ideal substrates for attempts to obtain hormone-regulated transcription in vitro.

4. In several cases, steroid hormones appear to inhibit specific gene transcription (97, 186). Can SR complexes also act as direct transcriptional repressor molecules or do they induce the synthesis of such repressors?

5. What is the primary structure of the steroid receptors? Which domains are important in hormone binding, DNA binding, and transcriptional activation? Are there other proteins that interact specifically with each of the steroid receptors? The relative paucity of highly purified receptor protein has made these questions unapproachable; however, literally dozens of laboratories around the world are applying recombinant DNA technology to this problem. Undoubtedly by the time this review is published, the first reports of receptor cloning will be upon us. This will facilitate not only a detailed analysis of receptor structure and function but will provide insight into receptor evolution,

ontogeny of receptors, and issues related to the control of receptor gene expression.

6. It is striking that the hormone sensitivity of any particular gene is generally confined to one or at most a few tissues. This is true despite the presence of functional receptors in many tissues. An area of great interest, and at this time little insight, is how tissue-specific expression of genes may determine their hormone responsiveness. A point worth noting is that the activation of genes by SR complexes may be quite distinct from developmental activation of genes (unless, of course, the hormone itself influences the developmental program). The analysis of genes that undergo a change from hormone insensitivity to hormone sensitivity during a well-defined differentiative program may be of significant utility in approaching this problem.

7. As alluded to earlier, many of the effects of steroid hormones on gene expression are due to the products of the genes directly activated by SR complexes. A challenge for the future is to unravel the complex network of events that leads to a highly orchestrated cellular response such as steroid-induced differentiation. It is particularly exciting to entertain the possibility that steroid hormones may provide a key to understanding not only how genes are transcriptionally activated in eukaryotic cells but, in addition, to understanding the control of developmental transitions and tissue differentiation at a molecular level.

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