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COMMENTARY

THE BIOLOGY AND MECHANISM OF STEROID HORMONE RECEPTOR INTERACTION WITH THE EUKARYOTIC NUCLEUS

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The precise molecular mechanism by which steroid hormones induce differential gene activity is not known. However, an ever-increasing body of inferential evidence suggests that steroids, including vitamin D, act via similar pathways to produce a similar set of general effects. With the exception of the glucocorticoids which produce both anabolic and catabolic effects, tissue responses to steroid hormones are characterized by overall increases in metabolism which include increased RNA, DNA and protein synthesis. However, even glucocorticoid-induced catabolic events require prior synthesis of RNA and protein. In some cases, the hormone-induced *de novo* synthesis of specific proteins has been described. These changes are initiated by the entry of small effector molecules, i.e. steroids, probably by passive diffusion, into a target cell. The effector molecules combine in a highly specific, noncovalent fashion with a limited number of cytoplasmic proteins termed receptors. The binding of the ligand promotes one or more conformational or enzymatic changes in the receptor molecule which then translocates to the nuclear compartment and affixes itself to acceptor sites located on the interphase chromosomes. As a result of this information transfer, a chain of nuclear events ensues. Although the initial cytoplasmic reactions may not be identical, thyroid hormone action is also characterized by such a sequence of nuclear events. The purpose of this review is to discuss the evidence which points to the above model of steroid hormone action, with particular emphasis on the nucleus as the primary site of steroid hormone action. In addition, we will offer some mechanistic models which may explain how this chain of molecular events could initiate transcription of specific genes.

Autoradiography

Injection of [^{3}H]estradiol into rats by Jensen and Jacobson [1, 2] resulted in the observation that the steroid was retained preferentially by some tissues. Similar experiments with other hormones revealed that estrogens were retained by the uterus and vagina [1–3]; progesterone was retained by the oviduct [4, 5]; testosterone was retained by the ventral prostate and seminal vesicles [6–8]; aldosterone was retained by the kidney and urinary bladder [9, 10]; and vitamin D was retained by the intestine [11]. Thyroxine was retained by nuclei of several tissues [12, 13].

To determine the subcellular distributions of steroid hormones, two lines of research have been pursued. After injections *in vivo* of labeled hormone, target tissues were isolated and various subcellular fractions prepared. The evidence suggested nuclear and cytoplasmic localization of radioactivity [14–17]. No specific binding to nucleoli was found [18]. Because of the inherent difficulty in preparing pure subcellular fractions and the possibility of isolation artifacts pointed out by Williams and Gorski [19], evidence from a second line of investigation has been extremely important in the unequivocal determination of the nucleus as the subcellular site of preferential hormone retention. The technical aspects of this second method, autoradiography, have been discussed by Stumpf [20]. Using autoradiography, several workers have observed the selective concentration of [^{3}H]17β-estradiol by various anterior pituitary cell nuclei [21, 22], certain hypothalamic nuclei [22, 23], and nuclei of uterine endometrial, stromal and myometrial cells [24–26]. Similar results have been obtained from autoradiographic studies of the intracellular localization of [^{3}H]aldosterone by the nuclei of kidney and bladder epithelia [27], and the nuclear accumulation of [^{3}H]progesterone by the uterus [28] and certain neurons of the hypothalamus [29]. Nuclear localization of steroid was shown to be specific in that the concomitant administration of the same steroid, unlabeled and in excess, blocked the nuclear accumulation of the tritium label. Co-administration of unrelated steroids did not block nuclear accumulation of labeled steroid by target tissues [29]. These experiments provided direct visual evidence that steroids are preferentially accumulated by nuclei of target cells.

Receptor-mediated steroid translocation to nuclei

The discovery of receptor proteins shed considerable light on the mechanism by which target cell nuclei selectively accumulate steroids. Classical pharmacology defines a receptor as a biological transducer which converts input information, in this case, circulating hormones, into a biologic response (e.g. increased RNA and protein synthesis). Biochemically, a receptor is a protein molecule which selectively

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binds a ligand in a saturable fashion and with high affinity ($K_d \sim 10^{-8}$ to 10^{-10}M). Jensen [30, 31] first postulated the existence of such a binding molecule for estrogen in the rat uterus. His observations were substantiated and extended by other researchers [14–17]. Similar steroid-specific binding proteins have been described for progesterone [32, 33], dihydrotestosterone [34–36], glucocorticoids [37, 38], aldosterone [39, 40], and most recently for vitamin D [41, 42] and thyroxine [13, 43–52]. These receptor proteins are cytoplasmic in the absence of hormone with one exception. Studies to date indicate that specific nuclear receptors exist for triiodothyronine (T3) [43–45, 52]. Moreover, these nuclear receptors are present in thyroidectomized animals [48, 49]. Although cytoplasmic thyroid hormone receptors have also been described [47, 50, 51], their relationship to the nascent nuclear receptors and thyroid hormone binders of serum has yet to be clearly resolved.

When whole tissues are incubated with labeled steroid under appropriate conditions *in vitro*, subsequent examination of nuclear and cytoplasmic fractions shows that much of the label has been accumulated by the nuclear fraction and that the cytoplasmic fraction has been depleted of its original capacity to specifically bind hormone [53, 54]. Alternatively, target tissues can be homogenized and binding studies carried out on purified cytoplasmic and nuclear fractions. In unstimulated target cells, selective high affinity binding of steroid occurs only in the cytoplasmic fraction. The addition of labeled hormone–receptor complexes to nuclei under appropriate conditions *in vitro* results in a selective nuclear accumulation of hormone–receptor complexes coupled with a concomitant decrease in cytoplasmic hormone–receptor complex. These observations are highly suggestive of a receptor-mediated nuclear accumulation of steroid.

Various model systems have been used to provide additional evidence that receptor proteins are required to effect specific binding of hormones to nuclei. Cytoplasmic receptors are present in lymphoblasts which are sensitive to the cytolytic actions of glucocorticoids [55, 56]. Conversely, cells resistant to the cytolytic actions of glucocorticoids contain few cytoplasmic receptor sites [55, 57]. Similarly, glucocorticoid-sensitive fibroblasts contain receptors [58, 59], whereas insensitive fibroblasts contain markedly diminished numbers of cytoplasmic glucocorticoid-binding molecules [59]. Diminished cytoplasmic levels of androgen receptor protein in the kidney of the adult Tfm mouse have been reported by Bullock *et al.* [60] and Ghering *et al.* [61]. Hepatic responses to 5α-dihydrotestosterone have been shown to be receptor mediated in a particularly nice study recently reported by Roy *et al.* [62]. A useful observation correlating the presence of cytoplasmic receptor proteins with tissue responsiveness to steroids has been made with respect to estrogen receptors in human breast cancers [63–65]. Tumors which contain significant titers of estrogen receptor stand a good chance of responding favorably to endocrine manipulations; those tumors with low receptor titers inevitably fail to respond to hormonal therapy.

Recently, evidence has been presented which suggests that deoxycorticosterone (DOC)-induced hypertension in the rat may be mediated via receptor proteins [66]. Sprague–Dawley rats with lesions in the lateral hypothalamus cannot be made hypertensive by DOC, whereas control rats are susceptible to DOC-induced hypertension. The Long Evans strain of rats is resistant to DOC-induced hypertension. Comparison of DOC receptor content in various regions of the brain shows that both strains of animals have similar levels of receptor in all areas of the brain except in the hypothalamus, where the Long-Evans animals contain significantly less receptor than the Sprague–Dawley strain. Thus, several lines of evidence from many different model systems indicate that steroid hormones bind to nuclear acceptor sites through reactions dependent upon specific receptors.

Quantitation of nuclear receptors by nuclear exchange

The nuclear exchange assay for estradiol has been a particularly useful methodological advance [67–72]. Injection of estradiol (E_2) *in vivo* drives cytoplasmic estrogen receptors into target tissue nuclei in a dose-dependent fashion. The extent to which nuclear translocation of cytoplasmic receptor has occurred is assayed *in vitro* by "exchange" of tritium-labeled estradiol with the cold estradiol complexed with receptor in the nuclei. Using this assay, tissues which did not contain cytoplasmic estrogen receptor were shown to be devoid of specifically bound nuclear E_2 in an exchangeable form [67].

The sensitivity of the nuclear exchange assay is such that variations in nuclear estrogen receptor complex are detected in response to physiologic variations in circulating estrogens. A 6-fold increase in nuclear-bound receptor occurs cyclically between metestrus and proestrus in the cycling female rat [68]. This variation in nuclear receptor closely parallels the secretion of estrogen during the estrous cycle [68].

Because the exchange assay depends upon the binding *in vivo* of unlabeled steroid–receptor complexes to nuclei, it was possible to examine the actions of various anti-estrogens. While evidence from other laboratories has documented the fact that some steroid antagonists function by binding to the cytoplasmic receptor and preventing nuclear uptake of the complex [73, 74], this cannot be the only mechanism of steroid hormone antagonism. Clark *et al.* [71, 72] have used the nuclear exchange assay to examine the anti-estrogenic actions of CI-628, nafoxidine and clomiphene. These three compounds apparently bind to uterine cytoplasmic estrogen receptor and are then translocated to the nuclear compartment in a normal fashion [71, 72]. Initial uterotrophic responses were observed, and these responses were maintained over prolonged periods of time. However, a subsequent restimulation or challenge with estradiol was completely ineffective. This result was explained by the observation that, in addition to the prolonged nuclear retention of estrogen receptor mediated by these anti-estrogens, cytoplasmic receptor resynthesis may have been blocked [72].

Cell-free binding studies

Measurement of nuclear binding or acceptor site capacity and binding constants has been achieved in several laboratories. Binding of hormone receptor complex to chromatin or nuclei appears to involve high affinity binding to several thousand sites per cell

Table 1. Literature summary of nuclear binding sites and dissociation constants (K_d)

Hormone	Model system	Binding sites	K_d (M)	Ref.
Estrogens				
	Rat uterine (immature) nuclei	7,700/cell	$2{\cdot}8 \times 10^{-10}$	[75]
	Rat uterine (ovex, mature) nuclei	No saturable binding		[76, 77]
	Rat uterus			
	Nuclei, *in vivo*	$2/10^7$ nucleotides	$2{\cdot}0 \times 10^{-10}$	[78]
	Chromatin, *in vivo*	$1/10^7$ nucleotides	$2{\cdot}0 \times 10^{-10}$	[78]
	Chromatin, *in vitro*	$2/10^7$ nucleotides	$2{\cdot}0 \times 10^{-10}$	[78]
	Rat uterus: physiological binding by exchange assay			
	Metestrus	930/cell	$2{\cdot}6 \times 10^{-9}$	[68]
	Diestrus	3,300/cell	$1{\cdot}6 \times 10^{-9}$	[68]
	Proestrus	4,700/cell	$1{\cdot}4 \times 10^{-9}$	[68]
	Estrus	1,000/cell	$1{\cdot}8 \times 10^{-9}$	[68]
	Rat uterus: DNA binding			
	Rat uterus DNA	1/base pair	$4{\cdot}6 \times 10^{-4}$	[79]
	Calf thymus DNA	1/base pair	$4{\cdot}6 \times 10^{-4}$	[79]
	E. coli DNA	1/base pair	$4{\cdot}6 \times 10^{-4}$	[79]
	Poly dAT	1/base pair	$4{\cdot}6 \times 10^{-4}$	[79]
	Reovirus RNA	No binding		[79]
	Chicken liver	1,000/cell	10^{-9}	[80]
Progesterone				
	Chick oviduct nuclei	8,600/cell	$1{\cdot}4 \times 10^{-8}$	[81]
	Chick oviduct chromatin	3,400/cell	$4{\cdot}0 \times 10^{-9}$	(*)
Androgens				
	Rat ventral prostrate			
	In vivo	2,000/cell		[82]
	Tissue incubation	2,000/cell		[82]
	Nuclei, *in vitro*	2,000/cell		[82]
	Rat ventral prostrate nuclei			
	In vitro	6,000/cell		[83]
Aldosterone				
	Rat kidney chromatin	600/cell		[40]
Glucocorticoids				
	Rat liver nuclei	10,000/cell	$1{\cdot}3 \times 10^{-9}$	[84]
	HTC cell nuclei	15,000/cell	$2{\cdot}1 \times 10^{-10}$	[85]
Thyroid hormones				
	Thyroxine			
	Whole GH_1 cell incubation	5,000/cell	$2{\cdot}6 \times 10^{-7}$	[45]
	GH_1 cell nuclear extracts	8,000/cell	$1{\cdot}3 \times 10^{-9}$	[52]
	Triidothyronine			
	Whole GH_1 cell incubation	5,000/cell	$2{\cdot}9 \times 10^{-8}$	[45]
	GH_1 cell nuclear extracts	8,000/cell	$1{\cdot}8 \times 10^{-10}$	[52]
	Rat (thyroidectomized) liver nuclei	10,400/cell		[49]
	Rat (euthyroid) liver nuclei	1·7 pmoles/g tissue	$6{\cdot}1 \times 10^{-8}$	[86]
	Rat (euthyroid or thyroidectomized) liver nuclei	5 pmoles/mg DNA	$4{\cdot}0 \times 10^{-10}$	[48]
Vitamin D				
	Rachitic chicks, *in vivo*	460/cell		[87]
	Rachitic chick intestinal homogenates, *in vitro*	2,500/cell	$5{\cdot}0 \times 10^{-9}$	[41]

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(Table 1). We have recently studied the kinetics of chick oviduct progesterone receptor interaction with nuclei [81] and chromatin.* The results were remarkably similar and suggest that the nuclear membrane does not play an essential role in the equilibrium-binding distribution of receptors. Moreover, we obtained essentially the same results using receptor purified to homogeneity by affinity chromatography [88*]. Oviduct contained more binding sites than other tissues, but the binding affinity varied little from tissue to tissue. High ionic conditions decreased nuclear binding capacity but not the binding affinity for steroid hormone receptors. Such a finding was consistent with chromatin conformation changes masking possible acceptor sites at higher salt concentrations.

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One surprising finding involved the elucidation of apparent nuclear binding site heterogeneity. In addition to a large number (~9000) of high affinity binding sites ($K_d \sim 10^{-8}$ M), Scatchard analysis suggested the presence of at least one other class of binding sites of even higher affinity. Because this very high affinity binding component was present in such low titers, it was difficult to quantitate binding parameters for it. The K_d approximated 10^{-11} M, and the sites per nucleus may have been on the order of a few hundred. The implications of nuclear binding site heterogeneity will be discussed in greater detail later in this commentary.

The extent to which DNA participates in the binding of hormone–receptor complexes to chromatin varies somewhat, depending upon the hormone studied, and has not yet been adequately explained. The regions of the genome to which aldosterone, glucocorticoid and estrogen receptors [75, 78, 89–91] bind are apparently much more susceptible to digestion by DNase than the corresponding regions to which progesterone receptors bind in the chick oviduct [92]. In the latter case, both the number of acceptor sites and the binding constant remain constant even with prior digestion of up to 60 per cent of the nuclear DNA content.* Moreover, neither pretreating oviduct chromatin with mung bean nuclease, which is specific for single strand DNA, nor incubation with specific antibody for single strand DNA blocked subsequent chromatin binding of progesterone receptors.† Thus, chick oviduct progesterone receptors do not bind to single strand DNA, or to double strand regions which are accessible to digestion by DNase I. These findings do not support Crick's general model for gene control which requires that regulatory macromolecules interact with single strand regions of the genome [93].

It seems difficult, if not impossible, for DNA to be the sole determinant of receptor binding to the genome. While it is true that not all tissues contain receptors, mixing experiments performed in cell-free systems have shown that receptors isolated from target tissues bind preferentially to target tissue nuclei and chromatin [82, 83, 92, 94, 95†]. Since within a given animal all tissues contain the same DNA, such a finding would require that specific regions of the DNA in nontarget tissues be masked by nuclear proteins or that nuclear proteins participate in a cooperative fashion with DNA in defining specific nuclear acceptor sites. Evidence in support of the first possibility comes from reports of saturable binding of hormone–receptor complexes to purified DNA [78, 90]. Baxter *et al.* [90] found that chromatin with receptors bound to it was more resistant to digestion by DNase, which suggested that receptors were covering up exposed DNA. Receptor interaction with DNA has even been shown to be hormone dependent [96]. However, except for the preliminary suggestion that unique and middle repetitive sequence DNA may be involved in receptor binding [97], no firm proof exists that specific acceptor sites are defined by a specific sequence of nucleotides. Indeed, glucocorticoid receptors apparently bind equally well to single and double strand homologous DNA as well as to *Escherichia coli* DNA [18]. Estrogen receptor binding to DNA has been similarly characterized [78, 79, 98, 99]. Yamamoto and Alberts [79] showed that estrogen receptors bind equally well to heterologous DNA and synthetic polynucleotides, but not to double strand RNA. The binding affinity these authors reported was much lower than that reported by other workers (Table 1). Thus, it would seem likely that chromosomal proteins play an active role in receptor interactions with the genome. Strong evidence in support of this hypothesis has been offered by two laboratories working independently on different hormone systems.

Puca *et al.* [100] have recently reported the identification of a high affinity nuclear acceptor site for the estrogen receptor of calf uterus. The apparent acceptor fractionated with the basic proteins and was present at levels five to ten times in excess of cytoplasmic receptors. Studies of receptor interactions with acceptor were carried out by means of affinity chromatography. These studies revealed that the interactions were influenced by salt and dependent upon hormone [100].

Our laboratories have previously implicated nonhistone (acidic) protein involvement in the nuclear acceptor site for the progesterone receptor of chick oviduct [101–103]. Several lines of reasoning suggest that acidic proteins are a logical choice to confer acceptor site specificity. First, they are a very heterogeneous class of proteins resolved by size, charge and immunological procedures. This contrasts with the relative homogeneity of the histones. Second, unlike histones, nonhistone chromosomal proteins turn over rapidly. Third, tissue specificity of nonhistones has been shown [104]. Fourth, specific changes in the nonhistone protein population of cells have been demonstrated in response to hormone administration [105, 106]. Finally, Spelsberg *et al.* [102, 103] have performed chromatin reconstitution studies which demonstrate that chromatin-associated receptor binding capacity can be transferred to nontarget tissue chromatin by transfer of a particular fraction of the nonhistone chromosomal proteins. Thus, it seems probable that the actual chromatin-associated acceptor sites consist of a DNA backbone which is structurally modified by chromatin-associated nonhistone proteins.

Nuclear binding of receptor is hormone dependent

Analogous to the receptor requirement for hormone accumulation by nuclei, there is a hormone requirement for nuclear retention of receptors. High salt extracts prepared from highly purified estrogen-primed chick oviduct nuclei contain little progesterone binding activity [53]. The same observation has been made with regard to the absence of receptors in nuclei prepared from immature rat uterus [24] and adrenalectomized rat liver [107]. Similarly, incubation of receptors *in vitro* with chromatin or purified nuclei in the absence of hormone results in little high affinity binding of receptor to the genome

* R. E. Buller, unpublished.

† R. B. Jafe, S. H. Socher and B. W. O'Malley, manuscript submitted for publication.

[82, 92, 108]. In contrast, significant quantities of receptors are retained by nuclei or chromatin when the incubation is carried out in the presence of hormone. Such studies strongly suggest an absolute hormone requirement for nuclear and chromatin binding of receptors. It is possible that, *in vitro*, receptors are distributed freely throughout both the cytoplasm and nucleoplasm but are only retained by nuclei in the presence of hormone. Such a distribution would suggest a hormone-induced modification of receptors which effects an alteration in the equilibrium distribution of receptors in favor of the nuclear compartment.

Receptor activation for nuclear binding

Virtually all steroid hormone–receptor complexes, including vitamin D receptor–hormone complex [109], undergo temperature-sensitive nuclear binding. Experiments under cell-free, low-salt conditions indicate very little receptor binding to nuclei or chromatin at 0° [84, 85, 89, 92]. In contrast, incubation of receptors with nuclei at 22–37° results in significant binding to nuclei or chromatin. This finding is consistent with the observation that incubation of hormone *in vitro* with tissue slices requires elevated temperatures (37°) for the nuclear localization of hormone to occur [24, 47, 110]. Several possible explanations for these observations may be offered. First, temperature may induce changes in cytoplasmic receptors which facilitate nuclear binding. Second, the nuclear uptake process itself may be influenced by temperature. Third, temperature may influence the availability of nuclear acceptor sites. Fourth, the interaction of receptor with acceptor may be facilitated by elevated temperatures. Finally, some combination of the above effects may occur *in vivo*.

Jensen *et al.* [111] were the first to observe that warming estrogen receptors in the absence of nuclei and subsequent incubation with nuclei at 0° completely removed the temperature requirement for nuclear binding. These receptors were termed temperature activated. We recently reported that, under similar conditions, the temperature requirement for progesterone receptor uptake by purified oviduct nuclei was only partially obviated by prewarming the receptor fraction [92]. Thus, temperature involvement in a subsequent step of nuclear binding of progesterone receptor was not completely ruled out. To define such a step, we studied the binding of receptors to nuclei at both 0° and 25°. Receptors activated by precipitation with ammonium sulfate were used because such a method removed free hormone and effected a simple, single-step partial purification [92, 112]. Most significantly, ammonium sulfate-activated receptor did not undergo subsequent temperature activation, so we had a fixed pool size for binding studies at both 0° and 25° [81]. Kinetic studies revealed the presence of more acceptor sites at 25° than at 0° [81]. Since these sites had the same apparent K_d values, they were assumed to be equivalent. While nuclear binding at 25° appeared to be more rapid than binding at 0°, this increase in apparent rate was due to the presence of twice as many acceptor sites at 25° than at 0°. Thus, the overall rate of the nuclear binding process showed minimal temperature dependency over the range of 0–25°. Such a finding is consistent with a diffusion limited uptake of oviduct progesterone receptors. This result contrasts with an active transport process suggested by other authors [113].

Both salt and temperature activation of estrogen receptor results in a conformation change which is detectable on high-salt sucrose gradients as a shift in sedimentation constant from 4S to 5S [114, 115]. Some authors note that this shift follows bimolecular kinetics and suggest it occurs by the addition of a subunit [98, 116]. However, Erdos and Fries [117] found no difference between the 4S unactivated cytoplasmic form and the 5S activated, or nuclear form of the estrogen receptor when analyzed by gel filtration under extreme denaturing conditions. Activated progesterone receptors do not sediment any differently from unactivated receptors in high salt [92]. Kalimi *et al.* [118] have made a similar observation with regard to glucocorticoid receptor activation. However, while gross conformational changes may not occur during progesterone and glucocorticoid receptor activation, more subtle electrostatic changes most certainly do occur. Heating hepatic glucocorticoid receptor for 30 min at 25° not only increases the receptor's ability to bind to nuclei and to DNA-cellulose, but also results in a dramatic shift in the receptor's isoelectric point from a pI of 7·1 to a pI of 6·1 [118]. Mainwaring and Irving [119] have reported that heating the DHT–receptor complex effects a similar shift in this receptor's isofocusing characteristics. Moreover, Milgrom *et al.* [108] found that increased receptor affinity for several polyamines following activation may be a general receptor property. We have recently observed that warmed progesterone receptors bind more readily to phosphocellulose [120]. Such an effect may also be induced by precipitation with ammonium sulfate. These results correlated remarkably well with an increased affinity of receptor for nuclear binding and with a shift in the receptor's sedimentation pattern on sucrose gradients run under low-salt conditions. In the latter case, there was a decrease in 6S and 8S material coupled with the appearance of 4S receptors. Such a finding suggests that receptor activation may merely involve disaggregation back to the original monomeric forms present *in vivo*. In any case, all of the above findings are consistent with an activation process *in vitro* which results in the exposure of increased regions of positive charge on the receptor.

While a large body of information has been accumulated which would tend to explain activation as a temperature or salt-induced conformation change in receptor molecules, there is also evidence that activation may be an enzymatic process. Puca *et al.* [121] have isolated and partially characterized a calcium-sensitive factor of calf uterine cytosol which may be involved in conversion of estrogen receptor from 5·3S to 4·5S in this system. A calcium-mediated alteration of the chick progesterone receptor has also been reported [122]. The significance of this observation is uncertain in that the altered form of the receptor is inactive in nuclear and chromatin binding *in vitro*.

A protease has also been described in human uterine cytosol which may be involved in estrogen receptor activation [123]. This protease, unlike the "Receptor Transforming Factor" of Puca *et al.* [121] described above, is a member of the trypsin group of

proteases because its action is inhibited by diisopropyl fluorophosphate, tosyl-lysine chloromethyl ketone and hydrolyzed benzoyl-arginine nitroanilide. Significantly, the human uterine protease described by Notides *et al.* [123] only acted on an estrogen–receptor complex. Such a hormone dependency would be required to explain temperature activation of estrogen receptors in light of the data presented earlier.

In summary, the important observation that the interaction of receptors *in vitro* with the genome is facilitated by elevated temperatures has led to publication of a large number of papers concerning "activation processes" which may be temperature, salt, and/or enzymatically mediated. It is essential to realize that activation is simply any process which facilitates the rate or extent of nuclear binding *in vitro*. It may have no significant relevance *in vivo*. Thus, one must beware of entanglement in elaborate activation mechanisms when activation may only be a disaggregation phenomenon experimentally required because of receptor preparation in hypophysiologic salt solutions.

Correlation of nuclear binding with biologic response

Studies which have attempted to correlate nuclear binding with biological responses have been inferential. Two recent lines of work have been strongly suggestive that such a correlation is indeed appropriate. Spirolactones are known antagonists of aldosterone. Normally, aldosterone promotes renal tubular readsorption of Na^+. However, in the presence of spirolactones, the antinatriuretic effects of aldosterone are greatly diminished. Marver *et al.* [73] have recently demonstrated that the spirolactone SC-26304 can competitively bind to kidney cytoplasmic aldosterone receptors, thus creating a spirolactone–receptor complex which is incapable of binding to nuclear acceptor sites. Thus, in the absence of the receptor–acceptor interaction, the antinatriuretic effects of aldosterone were blocked. These findings were exactly analogous to those reported by Kaiser *et al.* [74] with regard to the inhibition of nuclear binding of glucocorticoid receptors and the absence of glucocorticoid responses in the presence of cortexolone.

A second line of evidence which argues strongly for a correlation between nuclear binding of receptors and biologic response resulted from the studies of Anderson *et al.* [69], using nuclear exchange methods. The details of this method have been outlined already. These authors correlated estrogen-induced uterotrophic and growth responses with nuclear receptor content. They found that cytoplasmic receptor was present in excess of that required to produce maximal uterotrophic responses. In general, growth responses were proportional to the quantity of estrogen–receptor complex which remained bound to the nucleus for 6 hr and not to the amount of complex which was driven into the nucleus immediately after administration of large doses of estradiol *in vivo*. Analogous work with anti-estrogens [71, 72] showed that prolonged nuclear retention of anti-estrogen–receptor complex resulted in prolonged uterotrophic responses. Also, exchangeable nuclear estradiol has been shown to correlate with induction of a specific protein, IP [124]. Thus, nuclear retention, rather than just nuclear binding, of receptors probably determines cellular responses.

Cell genetic variants in hormone response

Recent work has demonstrated that even nuclear retention of hormone receptor complexes does not guarantee responses. Cell hybridization studies produced cells which contain glucocorticoid receptors indistinguishable from parental receptors [125]. Nuclear binding in the hybrid clones was also observed; however, TAT (tyrosine aminotransferase) induction did not occur. Such a finding suggests several possible explanations. Because glucocorticoids do more than induce TAT, production of hybrid clones may simply have resulted in the deletion of the TAT gene, leaving behind many other genes whose expression is influenced by glucocorticoids. Alternatively, the nuclear acceptor site whose occupance by receptor induces TAT may have been altered so that TAT becomes uninducible. Isolation of TAT mRNA and hybridization studies could differentiate between these hypotheses.

A most interesting finding has been reported by Lippman *et al.* [126]. They have cloned human and mouse leukemic cell lines which contain glucocorticoid receptors in amounts comparable to those of normal tissues. No unusual findings were made with regard to affinity and specificity of these receptors. Saturable high affinity nuclear binding of receptors was also demonstrated. Yet the cells were totally unresponsive to steroid administration. No changes in growth, macromolecular synthesis, amino acid uptake, or glucose utilization were detected. These results expanded upon an earlier observation by Gehring *et al.* [127] of a mouse lymphoma cell line, containing glucocorticoid receptors, which was unresponsive to the steroid. Using special techniques to isolate lymphoma cell variants resistant to killing by glucocorticoids [128], Sibley and Tomkins [129] have succeeded in characterizing cell lines whose steroid resistance can be attributed to each of the following three classes of defects: (1) absence of a cytoplasmic receptor; (2) deficient nuclear transfer of apparently normal cytoplasmic receptors; and (3) failure of reactions subsequent to nuclear localization of receptor–hormone complex. Yamamoto *et al.* [130] have gone one step further and shown altered DNA-binding properties of glucocorticoid receptors which could not be driven to the nucleus by glucocorticoid administered to whole cells in culture. Thus, presence of receptors is a necessary, but not sufficient condition for hormone responsiveness.

Steroid induction of protein synthesis

Steroid-induced proteins have been identified in several systems. Glucocorticoids specifically induce the production of tryptophan pyrrolase [131], tyrosine amino transferase [132, 133], and glutamine synthetase [134, 135]; estrogens induce a specific protein in the rat uterus [136, 137]; vitamin D causes increased synthesis of a Ca^{2+} transport protein [138, 139]; aldosterone-induced alterations of Na^+ adsorption apparently result from increased synthesis of aldosterone-induced proteins [140]. The study of estrogen-mediated synthesis of ovalbumin in the chick oviduct has shed much light on the mechanism of

induction of specific protein synthesis by steroid hormones. Two responses to the estrogen diethylstilbestrol (DES) have been studied. The primary response is elicited by injection of DES into newly hatched chicks and results in oviduct growth and differentiation toward cell types which produce ovalbumin. When these animals are withdrawn from daily DES injections, ovalbumin synthesis stops and the tissue shrinks in size. Re-stimulation with DES (secondary response) results in a much more rapid sequence of estrogen responses including ovalbumin synthesis.

Primary site of hormone action

While some attention has been directed toward methods of post-transcriptional control of steroid-induced protein synthesis [141], it seems likely that steroid hormones exert their primary effects at the level of transcription. Administration of aldosterone [142], glucocorticoids [143, 144], estrogen [145–147], progesterone [148], dihydrotestosterone [149, 150], vitamin D [151, 152] or thyroid hormones [153] under conditions which favor localization of hormones *in vivo* results in increased RNA synthesis.

A number of possible mechanisms can be offered which would explain steroid-induced increases in RNA synthesis. The activity of RNA polymerase may be directly modified by interactions with receptor–hormone complexes. New initiation sites for RNA polymerase may be created on the genome. Alternatively, the rate of RNA chain elongation may be greatly enhanced. To distinguish between these and other possibilities, it has been necessary to use various techniques *in vitro*. Ultimate proof of the mechanism by which steroids modify RNA synthesis will require the use of receptor purified to homogeneity. The step-by-step details of a hormone-dependent, tissue-specific increase in RNA synthesis can then be elucidated. To date, no model has been proven, although much exciting preliminary data have been accumulated. Two types of data exist: that which describes changes in nucleolar RNA polymerase activity (polymerase I) and that which describes changes in nucleoplasmic RNA polymerase activity (polymerase II).

The findings of Sajdel and Jacob [154] indicate that administration *in vivo* of a single dose of hydrocortisone to adrenalectomized rats results in a dramatic increase in nucleolar, but not nucleoplasmic, RNA polymerase activity assayed *in vitro*. They determined that hormone treatment resulted in increased enzymatic activity rather than increased amount of enzyme. Such a finding was consistent with a model of allosteric modification of nucleolar polymerase. However, their data do not necessarily imply that such an alteration is the primary event in steroid hormone action.

Some of the best studies on endogenous polymerase activity have been conducted by Davies and Griffiths [155–157] who incubated androgen receptors with prostatic nuclei and chromatin *in vitro*. They demonstrated a putative receptor-mediated, hormone-dependent, tissue-specific enhancement of prostatic nucleolar polymerase activity. More recently these investigators have also described increases in nucleoplasmic RNA polymerase activity [157]. This latter finding is consistent with work from other laboratories who have reported hormone-mediated specific increases in DNA-like RNA synthesis. A specific inhibitor of nucleoplasmic RNA polymerase, α-amanitin, blocks the induction of RNA synthesis by 1,25-dihydroxy-cholecalciferol in organ-cultured chick intestinal mucosa cells [158]. Zerwekh *et al.* [159] have reported a 2-fold increase in nucleoplasmic RNA polymerase activity within 2–3 hr of administration of an oral dose of 1,25-dihydroxy-vitamin D_3 to rachitic chicks. Mainwaring and Jones* report that similar increases in nucleoplasmic RNA polymerase activity in studies *in vitro* of androgen-stimulated rat prostatic chromatin are predominantly due to elongation of nascent RNA chains.

The antibiotic rifampicin binds to *E. coli* RNA polymerase molecules not involved in chain elongation [160]. Thus, under appropriate conditions [161–164], it is possible to further subdivide general increases in RNA synthesis between those due to rates of chain elongation and those due to increased template capacity. Tsai *et al.* [164] and Schwartz *et al.* [165] have recently applied these techniques to study the estrogen-induced increase in RNA synthesis in the chick oviduct [166]. Administration of estrogen *in vivo* to immature chicks results in an increase in RNA chain initiation sites on oviduct chromatin as measured with *E. coli* RNA polymerase under rifampicin challenge conditions, while the rate of chain elongation remains unchanged.

Using viral reverse transcriptase and purified ovalbumin mRNA, Harris *et al.* [167] have prepared highly radioactive copies of part of the gene responsible for ovalbumin mRNA synthesis. This can be an extremely sensitive probe for RNA-[^{3}H]cDNA hybridization and was, therefore, used to determine the ovalbumin messenger RNA content of oviduct cells during estrogen stimulation. They found essentially no copies of the ovalbumin mRNA in the unstimulated oviduct or in the oviduct of an animal which had been estrogen stimulated but subsequently withdrawn from hormone [167]. Moreover, readily measured increases in ovalbumin mRNA were detected as early as 30 min after secondary stimulation with estrogen. The absence of blocked or partially degraded ovalbumin mRNAs in unstimulated and stimulated-withdrawn animals suggested that estrogen induces *de novo* synthesis of ovalbumin message. Finally, the observation that *E. coli* RNA polymerase transcribes ovalbumin message from chromatin made from estrogen-stimulated animals but not from chromatin isolated from unstimulated animals strongly argues in favor of a derepression of template sites as a result of hormone treatment.† This finding is incompatible with the original model of post-transcriptional control which called for continous synthesis of mRNA produced from an "open gene". The intracellular concentration of this mRNA would then increase via subsequent steroid hormone control of its degradation [141]. Our data obtained in chick oviduct, however, are more consistent with a pure transcriptional stimulation.

* W. I. P. Mainwaring and D. M. Jones, manuscript submitted for publication.

† S. E. Harris, R. J. Schwartz, A. Roy, M. J. Tsai and B. W. O'Malley, manuscript in preparation.

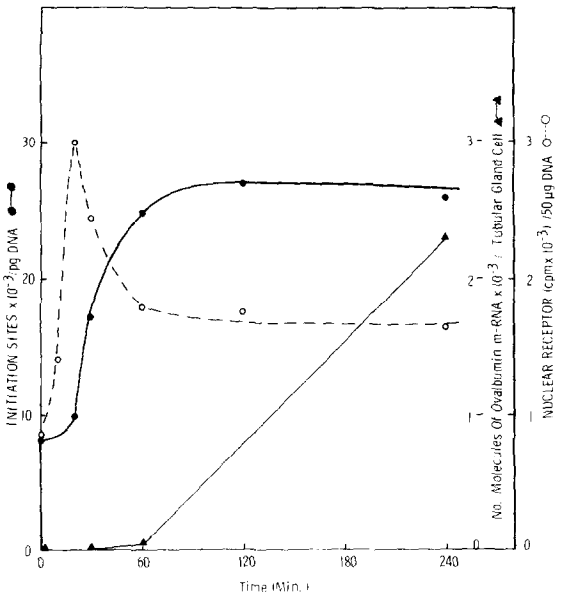

Fig. 1. Secondary response of chick oviduct to estrogen stimulation (with permission of S. Y. Tsai, M. J. Tsai, R. Schwartz, M. Kalimi, J. H. Clark and B. W. O'Malley, manuscript submitted for publication). Withdrawn chicks were injected with saline or DES (2·5 mg) and killed at the indicated times. Nuclear estradiol receptor was measured by the ^{3}H-estradiol exchange assay. Initiation sites for RNA synthesis were quantitated using *E. coli* polymerase under rifampicin conditions. Ovalbumin mRNA titers were determined by RNA hybridization to [^{3}H]cDNA.

Recently, we have established a temporal relationship between levels of nuclear bound estrogen receptor, polymerase binding and RNA chain initiation sites on chromatin, and ovalbumin message production in the stimulated-withdrawn chick given a secondary estrogen challenge [164, 165*]. This relationship is illustrated in Fig. 1. The figure shows a rapid increase in nuclear-bound estrogen receptor (measured by hormonal exchange methods) which peaks at 20 min after administration of DES. The number of chromatin-associated initiation sites per pg of DNA has also increased significantly by 30 min and continues to rise gradually thereafter. An increase in the ovalbumin message content of tubular gland cells begins after 1 hr. Thus, it would seem that hormone administration results in a cascade of effects in responsive tissues. The earliest response after receptor hormone binding to chromatin is initiation of mRNA synthesis [168]. This results from an increased availability of RNA polymerase binding and initiation sites. Subsequent increases in rRNA synthesis may occur via allosteric modifications of nucleolar polymerase [154], perhaps by the addition of newly resynthesized regulatory factors.

Models for receptor-mediated transcriptional control

Assuming that protein interactions with nucleic acids are determined intrinsically by primary structure, one may be justified in drawing certain parallels between transcriptional regulation in prokaryotes and steroid hormone effects in eukaryotes. Three specific regulatory mechanisms have been described in prokaryotes (see Refs. 169 and 170 for review): (1) direct regulation of RNA polymerase activity; (2) gene repression; and (3) gene induction.

The sigma regulatory molecule has been well characterized with respect to its influence on the increase in selective gene transcription by *E. coli* RNA polymerase [170, 171]. Certainly receptors may function by binding to eukaryotic RNA polymerase and thus signal the polymerase to bind to and transcribe a specific portion of the genome which had been previously shut down. This model is consistent with the increased RNA polymerase I activity observed by Sajdel and Jacob [154]. Moreover, it is interesting that Müller *et al.* [171] have recently obtained preliminary data suggestive of association between estradiol receptor and quail oviduct RNA polymerase I as determined by DEAE-cellulose chromatography, gel filtration and sucrose-gradient centrifugation. However, to date there is no conclusive proof of association between RNA polymerase and steroid hormone receptors. Moreover, the inferential evidence which has been accumulated [154, 171] suggests a relationship only with RNA polymerase I. Thus, we must look elsewhere to explain the increased RNA polymerase II activity which apparently precedes [168] the increase in polymerase I activity and is probably responsible for the transcription of new RNA sequences.

The catabolic gene activator protein (CAP protein) of *E. coli* is the best studied example of a positive regulatory factor responsible for induction of specific gene activity. Cyclic adenosine monophosphate (cAMP) binds specifically to the CAP protein to facilitate interaction between this regulatory receptor protein and DNA [172–174]. Activation of the catabolite sensitive genes (lac operon, gal operon and ara operon) is facilitated by this interaction. The role of cAMP and CAP can be replaced by agents such as glycerol, dimethylsulfoxide and other nonprotein agents [175, 176]. One characteristic of the DNA which is changed as a result of this interaction is the transition, or melt temperature (T_m), for the DNA duplex [176]. The transition temperature may be considered to be a measure of the activation energy for unwinding the DNA duplex. For the melting of the T7 early promoter region, the enthalpy of this conformation change has been measured. It is about 58 kcal/mole, consistent with a melting of about eight base pairs [177]. The energy of this reaction comes from the very tight binding of polymerase to the promoter region. Thus, by analogy, one many suggest that the energy released by the tight binding of the cAMP-CAP complex to promoter regions of affected operons is used to melt out the nearby DNA duplex, thus facilitating increased polymerase binding and transcription. That CAP binding results in increased polymerase binding sites is an established fact [178]. To carry the analogy one step further, one may surmise that steroid hormone receptor complexes interact with the eukaryotic genome in a similar fashion. However, the binding of steroid hormone receptors to DNA is modified by chromosomal proteins; thus local melt-out effects may not be reflected in finite overall differences in

* S. Y. Tsai, M. J. Tsai, R. Schwartz, M. Kalimi, J. H. Clark and B. W. O'Malley, manuscript submitted for publication.

T_ms for total nuclear DNA. Indeed, the temperature requirement for the formation of an initiation complex is less for chromatin than for DNA [164]. We may interpret this result to be an effect of the chromosomal proteins bound to DNA.

Perhaps some of the most critical chemical work on the nature of protein–DNA interactions has resulted from the study of the interaction of the lac [179–185] and gal repressor [172, 178, 186] proteins with DNA. Again, some striking similarities exist between these repressors and steroid hormone receptors. Both repressors are multimeric [174, 185]. Whether or not steroid hormone receptor proteins are multimeric *in vivo* is not yet resolved; however, the ability to permute the sedimentation constant of steroid hormone receptors *in vitro* [120, 187] is consistent with a capacity to form multimers. The progesterone receptor of chick oviduct has been shown to have a frictional ratio of about 20, consistent with the shape of a prolate ellipsoid [32]. Recently, the X-ray crystallographic study of lac repressor has shown that this tetramer is also quite asymmetric in shape [185]. perhaps a common mechanism describes the interacting of all these regulatory molecules with the genome.

Riggs *et al.* [183, 184] have found that the lac repressor interacts with both nonoperator and operator DNA. Similarly, we have found that chick oviduct progesterone receptors apparently bind to more than one class of nuclear acceptor sites [81]. The apparent association rate constant for lac repressor binding to operator DNA was such that the reaction was probably not diffusion limited [183]. We have preliminary data which indicate that the rate of interaction between progesterone receptor and the predominant class of nuclear acceptor sites ($K_d \sim 10^{-8}$ M) can be a diffusion-limited process. However, no association rate kinetics have been carried out on the very high affinity nuclear binding sites ($K_d \sim 10^{-11}$ M). It is possible that receptors bind to these very high affinity sites following one-dimensional diffusion along the chromatin away from the lower affinity sites.

The data presented above obtained from studies with prokaryotic organisms can be combined with the known details of steroid hormone receptor interaction with the eukaryotic genome and subsequent modification of transcriptional events to propose the following two models for receptor-mediated transcriptional control in eukaryotic organisms.

The first model, presented in Fig. 2, shows hormone (H) combining with cytoplasmic receptor (R_c). The

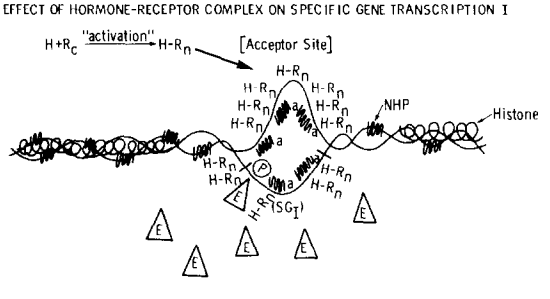

Fig. 2. Hypothetical model detailing steroid hormone-receptor modification of gene activity I.

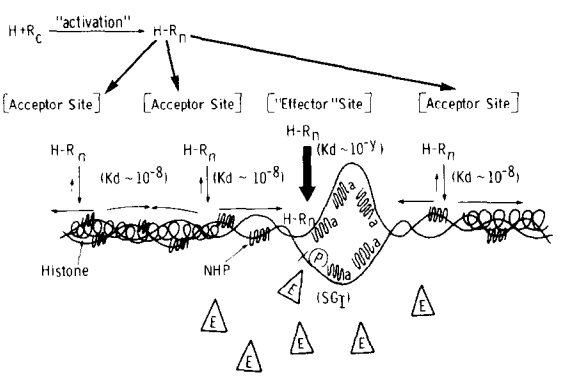

Fig. 3. Hypothetical model detailing steroid hormone-receptor modification of gene activity II.

complex somehow is altered by a process termed "activation", which facilitates intranuclear accumulation of the altered hormone receptor complex ($H\text{–}R_n$). The chromatin, consisting of DNA, histone and nonhistone proteins (NHP), contains specific acceptor sites for $H\text{-}R_n$. Perhaps specific acidic proteins (a) serve to concentrate the $H\text{-}R_n$ complexes at acceptor sites. Binding of $H\text{-}R_n$ to the chromatin induces a local melt out or unwinding of the DNA duplex which facilitates RNA polymerase binding (E) in the promoter region (P). Transcription through the structural gene (SG_I) ensues. The model would be independent of the exact number of receptors bound at each promoter site. Hormone response would likely be terminated by dissociation of the hormone from the receptor. Such a control mechanism is likely employed to turn off the hormone response because the dissociation constants for most hormone–receptor complexes ($K_d \sim 10^{-8}$ to 10^{-10} M) approximate the dissociation constants measured for nuclear binding of receptors (Table 1).

A second model for receptor interaction with the genome is shown in Fig. 3. Nuclear binding site heterogeneity is required by this model. Significantly, we have recently made the observation that progesterone-receptor binding to nuclei shows just such heterogeneity [81]. In this model, hormone–receptor complexes gain access to the nuclear compartment exactly as in the first model (Fig. 2). The high affinity of receptor for chromatin results in receptor localization at chromatin acceptor sites. These sites may be present in both target and nontarget tissue and consist of DNA and associated nonhistone chromosomal proteins. The receptor may then undertake a one-dimensional search along the chromatin until an actual "effector" site is reached where receptors may bind with a different affinity ($K_d \sim 10^{-y}$ M). The ensuing enzyme binding and specific gene transcription would be as in Fig. 2. The significance of this model lies in the fact that a three-dimensional search for effector sites located on a one-dimensional lattice is intrinsically longer than one conducted on the lattice itself [188]. Thus, the existence of thousands of moderately high affinity sites ($K_d \sim 10^{-8}$ M) would facilitate chromatin trapping of receptor, a three-dimensional search process, and ensure rapid localization to a very few effector sites ($K_d \sim 10^{-y}$ M) via

a one-dimensional random walk along the chromatin lattice. Thereafter, because of the extremely tight binding at the effector site, the additional receptors bound at the ($K_d \sim 10^{-8}$ M) lower affinity sites would no longer be needed to ensure continued hormone response. Such a model could explain the decrease in nuclear bound estrogen after 20 min of estrogen challenge, while continued ovalbumin synthesis occurs (Fig. 2). A specific "off mechanism" would have to be described to complete this model. Such an off mechanism would include the observation that the dissociation of hormone from receptor is prolonged when the complex is chromatin associated [189*]. Both of these models are really quite hypothetical at this time. The sole purpose in presenting them is to motivate investigators to think mechanistically in terms of how nuclear accumulation of steroid hormones elicits specific new RNA synthesis.

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