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Transcription Factors as Drug Targets: Opportunities for Therapeutic Selectivity

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Many traditional drugs target cell surface receptors. Medicinal chemists and pharmacologists have not ventured into the field of transcription regulation due to the fear that drugs that interfere with transcription regulation may not be selective or efficacious. The past 5 years have seen some exciting developments in the field of signal transduction in general, and transcription regulation in particular. Our understanding of mechanisms of regulated and basal transcription is advanced to a degree that it should be possible to selectively modulate a target gene directly. In this review we have argued that sufficient diversity exists in the combinatorial interplay of the transcription factors to offer opportunities for selective therapeutic intervention. We have focused our attention on transcriptional factors that play a role in three different therapeutic areas: osteoporosis, immune modulation, and cardiovascular diseases. Human estrogen receptor is considered as a model transcription factor. The role of estrogen in bone remodeling is discussed. Opportunities for tissue-specific modulation of estrogen receptors are described. For selective immune modulation, we have discussed the role of NF-AT (nuclear factors for activated T cells) transcription factors in interleukin-2 gene regulation. The last section focuses on the transcriptional mechanisms conferring tissue specificity in regulated expression of the apoAI gene, a major component of HDL, in liver. We have highlighted opportunities for rational development of transcription-based drugs useful for raising HDL plasma levels and atherosclerosis prevention.

Transcription Nuclear receptors Osteoporosis Estrogen receptor Immune suppression Atherosclerosis HDL

ALMOST 30 years have passed since Jacob and Monod proposed that gene expression can be differentially controlled by DNA binding regulatory proteins. However, not very many drugs have been discovered based on this concept. Instead many traditional drugs target cell surface receptors. These receptors transduce signals that modulate transcription of target genes, which in turn govern appropriate biological response. Sufficient information is now available regarding the transcriptional apparatus that it should be possible to selectively modulate a target gene directly. This, although conceptually attractive, poses major questions concerning the potential side effects and efficacy of the drugs: Will the extensive overlaps between membrane or cytoplasmic transducing systems allow for selective modulation of gene expression? Will the redundancies in these signaling cascades reduce the efficacy of such drugs? Clearly among these and related questions the issue of selectivity is most a critical and timely question in the field of "transcription factors as drug targets." In this review we argue that sufficient diversity exists in the combinatorial interplay of the transcription factors to offer opportunities for selective therapeutic intervention. The current status of basal and regulated transcription research is discussed briefly. A comparative analysis of nu-

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clear receptors with adrenoceptor pharmacology is performed in an attempt to highlight that selectivity is achievable while targeting receptors that appear to be ubiquitous. We have focused our discussion on selective topics in three therapeutic areas: osteoporosis, immune modulation, and cardiovascular diseases.

EUKARYOTIC TRANSCRIPTION

A brief overview of the transcription apparatus is warranted before we discuss transcription factors as drug targets. For a detailed discussion of the basal and regulated transcription the reader is referred to reviews by Kingston and Green (1994), Struhl (1993), and Parker (1993). The eukaryotic transcription apparatus is composed of protein factors that are generally divided into two groups. The first group is composed of general transcription factors that are necessary for accurate initiation of transcription by RNA polymerase II [i.e., TATA binding protein (TBP), TFIIB, and others].

The second group of transcription factors, generally called "activators," regulates the function of general transcription factors. The term "activator" is a misnomer because in a different context an activator may also suppress expression of a target gene. A more appropriate term often used in the literature is "transregulator." The basal level of transcription can be affected by transregulators that bind to upstream sequences in most, if not all, cases. Generally transregulators are composed of a DNA binding domain that recognizes specific DNA sequences and a transcription regulatory domain that communicates with the basal transcription apparatus. Typical examples of transregulators are the steroid receptors, NFκB, NF-AT, and AP1.

Regulated Transcription

A successful design of a drug that modulates transcriptional activity would require an adequate understanding of transcription control mechanisms. It is therefore essential that the target transcription factor and its role in the transcription apparatus are reasonably well understood before embarking on the drug discovery program. Control of transcription is primarily exercised at the level of initiation of mRNA synthesis. In most cases initiation begins 20–30 nucleotides downstream of an A/T-rich sequence referred to as the TATA box. The TATA box is a hallmark of most genes and it is involved in binding of the TATA binding protein (TBP), the first step in transcription initiation. Although TBP can bind the TATA sequence on its own, it normally exists as a part of a multiprotein complex referred as TFIID. TFIID is composed of about 10 proteins called TAFs. Following binding of TFIID to the TATA box, additional general transcription factors TFIIB, TFIIA, and RNA polymerase II are recruited to establish a preinitiation complex (for review see Kingston and Green, 1994).

What determines the rate of association of these transcription factors with the TATA box, the stability of the preinitiation complex, and the efficiency of transcription initiation? It is believed that transregulators play a critical role in the above processes and are the governing forces that determine activation or suppression of the target genes (Kingston and Green, 1994). It has also been argued that chromatin structure plays an important role in determining which genes are poised for transcription (Croston and Kadonaga, 1993). In addition to promoting the stability and recycling of the preinitiation complex, binding of transregulators to DNA may alter the chromatin structure that in turn facilitates transcription initiation. In addition to transregulators that influence transcription by binding to specific DNA sequences, biochemical and genetic studies have shown that another class of transcription factors that do not bind DNA mediate activation of genes by bridging the activation domain in transregulators with the general transcription machinery (Berger et al., 1992). These transcription factors are named adapters, coactivators, or transcription intermediary factors. Thus, different activators may function in different modes. It is known, for example, that interaction of TFIID with the TATA box and interaction of TFIIB with the TFIID promoter complex can both be slow steps (Kingston and Green, 1994). The acidic activator Gal4-AH was found to increase the rate of formation of a functional, promoter-bound TFIID/TFIIA complex (Wang et al., 1992), whereas the activator LSF was found to increase the rate of formation of promoter-bound TFIID/TFIIA/TFIIB complex (Sandseth and Hansen, 1992). Furthermore, the Gal4-VP16 activator physically associates with TFIIB to bring about a strong increase in target gene transcription (Colgan et al., 1993). These, as well as other studies, demonstrate that an increase in the rate of preinitiation complex formation has profound impact on the overall rate of transcription (Kingston and Green, 1994). Similarly, a herpes virus activator protein, ICP43, has been shown to interact with TBP/TFIIB in the absence

of DNA, suggesting that certain activators can influence the basal transcription machinery in the absence of DNA binding (Smith et al., 1993).

In general, eukaryotic genes can assume three distinct transcription states: silent, basal, and activated. Silent genes may be activated by removing repressors from the promoter sites. Nucleosomal histones, as well as histone H1, repress the basal level of transcription, thus creating a silent state. Activators dislodge nucleosomes, thereby promoting activation of transcription (Croston and Kadonaga, 1993). Under these circumstances the activators are playing the role of "antirepressor." A growing family of proteins that interact with the general transcription machinery to suppress transcription have been described (Drapkin et al., 1993). One of these repressor proteins, called Dr2, once cloned, turned out to be DNA topoisomerase I (TopI) (Merino et al., 1993). In the absence of an activator Dr2 acts as a repressor by binding to TBP. However, in the presence of activators, Dr2/TopI is translocated to the transcription elongation complex to remove superhelical turns created by the elongating transcription complex. Thus, Dr2/TopI is considered as a general transcription factor because its function is required by many transcribing genes.

A frequently raised question is whether general transcription factors can act as drug targets. Topoisomerase I inhibitors were being used as anticancer agents before a direct role for TopI was recognized in the transcription process. Currently, two derivatives of the TopI inhibitor camptothecin, irinitecan (Kunimoto et al., 1987) and topotecan (Kingsbury et al., 1991), are in late stages of clinical trial against metastatic solid tumors (Burris et al., 1992). These compounds have acceptable toxicity profiles even though their target may be a general transcription factor.

Targeting Transcription Factors

A large volume of data is available on the gross anatomy of transcription factors and their interaction with the basal transcription machinery. It is perhaps fair to say that precise details of the molecular events are not well understood. However, we do believe that current information is sufficient to embark on targeting transcription factors as drug targets. Among several transcription factors, mechanisms of the nuclear receptors in the steroid/retinoid family are fairly well advanced. The human retinoic acid receptor α (RARα) acts as a repressor in the absence of its ligand in mouse L cells, whereas in CV1 cells it acts as an activator (Baniahmad et al., 1992). Tissue-specific regulation by regulatory proteins is mediated by specific response elements present in the target genes, and by general transcription factors recruited to the promoter site. As mentioned above, the role of adaptors is that they act as bridges between the general transcription factors and the activation domain of the regulator. As the mechanistic basis of the transcription may vary between the three phases of transcription (i.e., silent or suppressed, basal, and activated), it begs the question as to what we mean by activation of transcription. True activation is defined as the ability of an activator to stimulate transcription of the target gene above the suppressed or basal level of transcription. Conversely, a transregulator may suppress the activated state of transcription by disrupting the bridge between the activator domain and the general transcription factors.

How does ligand binding to the nuclear receptors regulate transcription? Receptors for thyroid hormone (TRβ), retinoic acid (RAR), and vitamin D (VDR) are located in the nucleus, bound to their cognate response elements (Parker, 1993). Thyroid hormone receptor β is thought to interact with TFIIB (Baniahmad et al., 1993). In the absence of thyroid hormone, the receptor acts as a repressor. The ligand binding domain (LBD) of the receptor interacts with the N-terminal domain of the TFIIB in such a way that the "activation bridge" that connects TFIIB with TFIIF (30-kDa subunit), required for chain elongation, is disrupted (Baniahmad et al., 1993; Ha et al., 1993). Thus, TRβ acts as a repressor by interfering with the basal transcription machinery. Binding of the hormone to the LBD alters the nature of TRβ–TFIIB interaction, establishing the "bridge" with the basal transcription machinery that leads to the promotion of transcription. The suppression model states that ligand-dependent, receptor-mediated transactivation may be a passive mode of transcriptional activation. Do all members of the steroid receptor family regulate transcription in a similar fashion? Evolutionarily advanced receptors [e.g., progesterone receptor (PR) and estrogen receptor (ER)] exist in an inactive state in the cytoplasm, complexed with HSP90, P56 (an FK506 binding protein, i.e., immunophilin), and other chaperonins (Renoir et al., 1994). Ligand-bound receptors are translocated to the nucleus. The ER and PR contain at least two activation domains that are engaged in ligand-dependent activation of transcription (see the section on Estrogen Receptor and Parker, 1993). It therefore appears that in ER and PR the ligand plays a more

active role, first in translocation of the receptor and then modulation of transcription (see below).

Recognizing the modular nature of transcription factors, it is possible to reconstruct a transcription factor cell-based assay in a heterologous system such as yeast (Metgzer et al., 1988; Lyttle et al., 1992; Ohashi et al., 1991). As mentioned above, although substantial information on how activators and repressors modulate basal transcription exists, detailed understanding of the molecular interaction involved is limited. What is the role of chromatin structure in regulation of transcription? How does a single transcription factor perform different regulatory functions (repressor and activator) on different target genes? Hypothetical answers to some of these questions are offered in the next section.

DIVERSITY IN COMBINATORIAL INTERACTION OF TRANSCRIPTION FACTORS

Eukaryotic promoters contain a variety of DNA elements, indicating that regulation of transcription of a target gene is a function of various factors working in concert with each other to promote transcription. How does one transcription factor recognize and differentially regulate the target genes? Another part of the same question is: how can we achieve therapeutic selectivity by targeting a transcription factor that may be present in several tissues? It turns out that nature has devised remarkable homo- and hetero-multimeric interactions between the transcription factors of the same family. A combinatorial interplay between these proteins can produce "mosaic" transcription complexes that are unique for a given target gene, and hence amenable to therapeutic intervention.

We have chosen RXRα as a member of the steroid/retinoid family to illustrate several commanding interactions that can dramatically increase the combinatorial possibilities of its function (Table 1). The greatest amount of diversity is obtained when all four variations (A, G, C, and T) of a single consensus sequence are computed (see legend for Table 1 for details). Not all possible interactions listed in Table 1 have been documented in the literature as of yet. In total we estimate that more than 373,248 different unique interactions are possible for a single RXRα. This may even be a conservative estimate, as we have not included other known heterodimeric partners. Also promoter context effects that can contribute greater diversity have not been included. The reader is referred to a review by Giguere (1994) on details of the RAR-RXR family of interactions.

Previous studies have shown that the DNA recognition code for a number of nuclear receptor family is based on the spacing between the direct repeats of the response elements, called the 3.4.5 rule. The original data suggested that the preferred spacing is three nucleotides for TR, four nucleotides for VDR, and five nucleotides for RAR (Umesono et al., 1991). Although this rule has been very helpful in developing our understanding of combinatorial interactions, it is apparent from recent data that the actual recognition code is more complex than was originally conceived. The elegant studies of Gronemeyer and colleagues (Zechal et al., 1994a, 1994b) suggest that multiple homo- and heterodimeric interactions between RXRα and other molecules with respect to half site orientation, sequence, and the promoter environment will lead to different transcription outcomes. Similarly, we believe that human estrogen receptor interaction with tissue-specific ancillary transcription factors and that of NF-AT can lead to a multiplicity of interactions that are open to selective therapeutic intervention.

LESSONS FROM ADRENOCEPTOR PHARMACOLOGY

As we move into the development of a new class of drugs that target transcription factors, we believe that there are analogies and lessons to be learned from classic pharmacology. A particular lesson is that it is possible to design selective antagonists against receptor subtypes that may be highly conserved in the body. It is important to pay tribute to our colleagues in pharmacology as drug discovery moves from cell membrane receptors to nuclear receptors. Historically, adrenergic pharmacology has guided our thinking about drug discovery and drug action (Vanhoutte et al., 1994). This is obvious as adrenoceptors play a fundamental role in central and peripheral actions of noradrenaline, a neurotransmitter, and adrenaline, an adrenal medullary hormone. Adrenoceptors α_1, α_2, β, and their respective subtypes are all G-protein-linked receptors (Bylund, 1992).

A plethora of drugs that interact with these and other receptor subtypes play a major role in treatment of diseases such as hypertension, congestive heart failure, asthma, depression, and prostatic hypertrophy (Black and Prichard, 1973; Vanhoutte et al., 1994). β_1-specific agonists have been used to treat congestive heart failure (Hieble and

TABLE 1
COMBINATORIAL INTERACTIONS BETWEEN RXRα AND OTHER TRANSCRIPTION FACTORS

Nature of Interaction	Type	Possible Variability	Subtotal
Response element spacer variability	0–5 spacer	6	6
Heterogeneity of the sequence at each half-site	One base substitution in a 6-base half-site*	>324 possible pairs of half-sites	>1,944
Orientation of the half-sites	Direct repeats →→ Inverted repeats →← Everted repeat ←→	3	>5,832
Heterodimeric partners	RARα, β, γ TRβ, TRα PPARα, β, γ VDR, ARP COUP Other orphans (5)	16	>93,312
Ligand	Ligand dependent/ independent	2	>186,624
Posttranslational modification	Phosphorylation (+/−)	2	>373,248

Number of combinatorial interactions between RXRα and heterodimeric partners. The sole purpose of this scheme is to highlight the number of possible heterodimeric interactions of RXR. The consensus response element used in this example is of DR5 type, direct repeat with five spacer, AGGTCAnnnnnAGGTCA (Parker, 1993; Giguere, 1994). Although all possible variations in the spacer sequence and the DNA sequence of the half-sites have not been fully documented as yet, we have taken the liberty to speculate on possible heterogeneities that may exist on different levels. Ligand-dependent and ligand-independent states of RXR may contribute to further regulation of a given heterodimer. We have not included the consequences of dominant negative receptors that may recruit RXRα to a promoter site and contribute to the ever-increasing diversity of RXRα. We have added only five orphan receptors to the list (Parker, 1993). We have not included various isoforms of the receptors that could further increase the diversity of the interaction.

*Assume all one mutant variations of a *single* consensus response element

Ruffolo, 1991). The β_1 adrenergic receptor is encoded by a single gene (Frielle et al., 1987), yet we find a remarkable number of agents that have been developed against one receptor subtype that are clinically useful for several different pathologies (Hieble and Ruffolo, 1991). This is, of course, possible by virtue of tissue-restricted expression and the microenvironment of the receptor signal transduction system. It is important to note that drug discovery in adrenergic pharmacology was facilitated by the synthesis of new pharmacological agents that preceeded the discovery of receptor subtypes. Most recently it has been shown that the cloned α_{1c} receptor subtype is responsible for smooth muscle contraction in human prostate as well as muscle contraction in peripheral nervous system (Forray et al., 1994). There is an indication that a prostate-specific α_{1c} antagonist could alleviate the problem of benign hypertrophy of prostate. Tissue-selective α_{1c} antagonists are poignant examples of the single receptor as a target of more than one pathology. Thus, drug discovery experience with adrenoceptor pharmacology suggests that it is possible to design selective agonists and antagonists for highly conserved forms of receptors. Modern day molecular pharmacologists should take solace from these lessons and hope that more specific agents that interfere with or enhance specific transcriptional processes may be possible. In fact, we will go one step further and propose that if a certain adrenoceptor expression in a tissue is responsible for the pathology, then we should consider regulating the receptor production at the transcription level.

OSTEOPOROSIS

Osteoporosis means "porous bone condition" and results from the loss of normal, mineralized bone. Osteoporosis is the major cause of bone fracture in the aging population and particularly in postmenopausal women. Although effective therapy for the prevention of bone loss (hormone

replacement therapy) exists, patients are not diagnosed until the later stages of the disease, at which point the therapy is much less effective (Fitzpatrick, 1989). Osteoporosis is a major health care problem, and epidemiologic studies reveal that it is associated with enormous cost to the health care system, and greatly increases morbidity and mortality in older people (Favus, 1990). The human skeleton continues to increase in bone mass until puberty and peaks at age 35. The relationship between bone formation (osteoblast function) and bone resorption (osteoclast function) changes after age 30. An imbalance between osteoblast and osteoclast function that is triggered at midlife is the underlying cause of osteoporosis (Horowitz, 1993). Women are much more prone to osteoporosis than men, indicating the role of sex steroids in the etiology of the disease. Precise cause of the pathogenesis of osteoporosis is not understood, but both increase in osteoclastic resorption and decrease in osteoblast function, coupled with aging, results in imbalance and the bone loss. Genetic factors may play an important role in susceptibility to an early onset of osteoporosis. It has been suggested that allelic variations in the gene encoding for vitamin D receptor can be used to predict the differences in bone density, suggesting a role for vitamin D in osteoporosis (Morrison et al., 1994). Bone loss in women is directly related to estrogen status and thus far estrogen therapy is considered to be the most effective form of treatment (Lindsay et al., 1980). In this section we will briefly review the role of estrogen in osteoporosis and consider estrogen receptor as a prime target for bone-specific receptor ligands that act as anti-osteoporotic agents.

Role of Estrogen in Osteoporosis

Estrogen clearly prevents the rapid loss of bone that occurs at menopause (Lindsay et al., 1980). The critical question is whether estrogen targets osteoblasts to promote its bone-forming function or inhibits an osteoclast's ability to resorb the bone. Both osteoblasts and osteoclasts have been shown to contain estrogen receptors (Komm et al., 1988; Spelsberg et al., 1993). Healthy bone mass reflects a careful balance between osteoclast bone resorption and osteoblast bone formation. Estrogen possibly tilts this balance in favor of osteoblasts (see below) (Horowitz, 1993). In addition to preventing rapid bone loss in menopausal women, other beneficial effects of estrogen have been noted. Estrogen therapy, or more commonly known as hormone replacement therapy (HRT), is associated with reduction of hot flashes and reversal of vaginal atrophy (Bettendorf et al., 1993). Reduction in morbidity and mortality from coronary heart disease following HRT is the basis of a large clinical trial to test the role of estrogenic compounds in a number of the above-mentioned pathologies. With the remarkable number of effects of estrogen in the human body, the estrogen receptor emerges as the most exciting target for tissue-selective drug design in the field of nuclear receptors. The most recent clinical studies suggest that estrogen not only inhibits bone resorption but also leads to a net increase in the bone mass (Christiansen, 1993). This study suggest that osteoblasts or their precursors may be the targets of estrogen action. The estrogen receptor gene knockout mouse is viable and studies suggest that the females are infertile with no detectable response to estradiol (Lubahan et al., 1993). Subsequent studies on the ER mutant mouse suggest that there is 25–30% decrease in the bone mass (Korach, 1994, unpublished results). A definitive role of ER in bone remodeling appeared when an estrogen receptor-disruptive mutation identified in a young man, who suffered from osteoporosis, increased bone resorption (Smith et al., 1994). These studies confirm that estrogen has a direct effect on bone remodeling, and there is little doubt that the receptor is the key target for osteoporosis.

Because any osteoporosis therapy is likely to be for long-term duration, and given the endometrium and breast cancer risk associated with estrogen therapy, a bone-specific estrogen modulator is highly desirable. Current understanding of the mechanism of estrogen receptor function is fairly advanced, and it should be possible to identify truly bone-specific, CNS-specific (hot flashes), and cardioprotective ER-modulating agents.

Tissue-Selective, Estrogen-Specific Agents

The estrogen receptor is a member of the zinc finger, nuclear receptor family (O'Malley, 1990; Parker, 1993; Giguere, 1994). The estrogen-bound receptor stimulates transcription by interacting with estrogen-responsive elements (ERE) of target genes. How does ER modulate transcription? Molecular details of this process are not known; however, a large amount of data has been collected since it has become possible to transfect the ER gene into a variety of tissues and ligand-dependent transcription has been established, including in yeast (Metzger et al., 1988; Lyttle et al., 1992). ER contains at least two activation domains. AF1 is located near the N-terminal region. The second

activation domain, AF2, is present in the ligand binding domain in the C-terminus of the protein (Tora et al., 1989; Berry et al., 1990). Several agonists and antagonists for ER have been described. Tamoxifen, a well-known ER antagonist, has been used against breast cancer for several years (Jordan and Murphy, 1990). Appearance of tamoxifen-resistant tumors has shown that tamoxifen does not act as a pure antagonist for ER (Jordan and Murphy, 1990). A new class of agents that completely block the ER function (i.e., ICI 164,384) (Fawell et al., 1990) are also undergoing clinical trails (Wakeling et al., 1991). All these compounds compete for the estradiol binding site on the receptor. Binding of different ligands to the receptor affords a unique receptor structure that is distinct in molecular details (Reese and Katzenellenbogen, 1991; Pakdel and Katzenellenbogen, 1992).

It is convenient to organize the ER-modulating agents into three groups before we discuss the mechanisms of tissue-specific action of these compounds. The first class of compounds (I) includes true agonists, exemplified by 17β estradiol. Class II is composed of agents that demonstrate partial agonist activity or behave as mixed-function molecules, depending upon the tissue. Tamoxifen is the prime example of this class of drugs. Pure antagonist will fall into our group III of compounds; ICI 164,384 is the prime example of this group.

In general, the function of a transcription factor depends on three elements: 1) nature of the promoter, 2) tissue-specific basal transcription factors present in the cell, and 3) ligands (for references see below). Tamoxifen behaves as an antagonist in MCF-7 cells and as a partial agonist in avian and yeast cells (Berry et al., 1990). The class II modulators, like tamoxifen, allow the receptor to bind to the ERE. Tamoxifen-bound ER is unable to use its AF2, and the transactivation function in avian and yeast cells is conveyed by AF1 (Berry et al., 1990; Tora et al., 1989). What is the basis for the lack of AF1 function in MCF-7 cells? Deletion and mutagenesis of ER in animal cells and yeast have shown that the AF1 and AF2 are distinct activation domains; they can work independently or synergistically. It has been proposed that different classes of transregulators interact with basal transcription machinery via adapters that are tissue specific (Tasset et al., 1990). MCF-7 cells presumably lack the adapter that bridges the AF1 to the basal transcription machinery. Thus, one of the ways of selective regulation of ER function is mediated by selecting tissue-specific basal transcription factors.

Another example of cell context-dependent modulation of the ER also comes from work on MCF-7 cells that were treated with isobutyl methylxanthine plus cholera toxin to increase the intracellular levels of cAMP, up to 10-fold (Fujimoto and Katzenellenbogen, 1994). Increase in cAMP potentiated the effect of estrogen by 250%, and the tamoxifen antagonist effect was blunted to the extent that tamoxifen became an effective agonist in these cells. An intriguing aspect of these studies is that an antagonist/agonist switch in these cells is observed with the class II agents (i.e., tamoxifen) whereas ICI 164,384 remained a pure antiestrogen (Fugimoto and Ketzenellenbogen, 1994). Also, cAMP-dependent activation of transcription by tamoxifen was observed with several, but not all, estrogen-responsive promoters examined in this study. Whether the enhancement in transactivation or antagonist/agonist switch is due to increase phosphorylation of the receptor or another ancillary transcription factor that is part of the basal transcription machinery remains a matter of speculation. These results may also explain, in part, the molecular basis of breast cancer growth that is independent of tamoxifen (Jordan and Murphy, 1990). The key lesson that emerges from these studies is that not only the effect of a drug is selected by the state of cells but it is also dependent on the type of estrogen responsive promoter.

In another study simple ERE or complex C3 estrogen-responsive promoters were studied in monkey kidney fibroblasts (CV1), human hepatocellular carcinoma cells (HepG2), and human breast cancer cells (HS578T). Using estrogen, tamoxifen, and naftoxidene, these studies confirm that class II estrogenic agents are partial agonists and different activation functions of ER are operating in a cell and in a promoter-specific fashion (Tzukerman et al., 1994). Similarly, it has been shown that the ability of tamoxifen to act as an ER agonist in pituitary cells is restricted to several, but not all, estrogen-responsive genes (Schull, 1992).

In a series of comprehensive studies Pilat et al. (1993) examined the transcriptional regulation of estrogen-responsive cathepsin D and pS2 genes in MCF-7 cells by structural analogues of estrogens. 2-Hydroxyestratrien-17β-ol was capable of stimulating the pS2 mRNA but it did not have any effect on cathepsin D mRNA. 1-Hydroxyestratrien-17β-ol actively elicited both the mRNAs, whereas 4-hydroxyestratrien-17β-ol did not stimulate the synthesis of either of these genes (Pilat et al., 1993). Use of the same analogues on classic vitellogenin synthetic ERE did not demonstrate any selective regulation of estrogen-responsive transcription. In this case ER-mediated function was simply

correlated with the affinity of the ligand to the receptor (VanderKuur et al., 1993a, 1993b). These studies clearly argue that success in discovering tissue-selective agents requires the study of complex promoter elements in homologous tissues.

What is the role of pure antiestrogens in modifying the pathologies other than breast cancer? Unlike class II antiestrogens that do not interfere in binding of ER to ERE, the ICI 164,384 binding to ER impairs the receptor dimerization, which in turn inhibits its binding to ERE (Fawell et al., 1990). Hence, we define pure antiestrogens as class III molecules that totally impair estrogen receptor function. Although the clinical data are unavailable on the bone remodeling effects of pure antiestrogens, we suspect that these compounds may promote a similar situation as seen in ovariectomized women.

Estrogen mimetics that promote bone formation are strictly defined as agents that target osteoblast and osteoclast and spare breast and endometrial tissues, and do not interfere with lipoprotein profile (cardioprotective effects). How can we discover such estrogenic compounds that fulfill the criteria outlined above? As discussed in Table 1, we believe that the structure of a transcription complex in the context of a promoter is unique. In this regard the role of estrogen in modulation of cytokines and growth factor synthesis has become evident. Different theories have been presented that implicate production of TGFβ by osteoblast in response to estrogens (Oursler et al., 1991; Knabbe et al., 1991). Secretion of TGFβ leads to inhibition of osteoclast function and thus bone resorption. An alternate theory suggests that an estrogen-depleted state activates production of IL-1 and TNFα, which stimulate osteoclast formation (Horowitz 1993). Osteoblast-like cell lines activated with IL-1 and TNFα secrete IL-6 and the treatment with estrogen inhibits IL-6 production (Girasole et al., 1992). These finding are consistent with the observation that osteoblast cells have functional ER (Komm et al., 1988). We believe that employment of growth factor and cytokine promoters as targets for ER, in the context of homologous cells, will yield tissue-specific agents with minimal or no side effects.

SELECTIVE IMMUNE SUPPRESSION

The immune system is a double-edged sword. Stimulation of the immune system is required to fight a variety of pathogenic attacks. On the other hand, selective immune suppression is desirable to maintain transplanted organs and to manage autoimmune diseases, including psoriasis, rheumatoid arthritis, nephrotic syndromes, and other inflammatory disorders. This section will focus on strategies for selective immune suppression to prevent rejection of transplanted organs.

Presentation of foreign antigens to T cells initiates a cascade of events that culminate in tissue rejection. T-helper 1 (Th1) and T-helper 2 (Th2) cells are fully differentiated CD4+ T cells that predominantly promote cellular and humoral immune responses through secretion of distinct cytokines (Fraser et al., 1993; Mosmann and Coffman, 1989). Th1 cells produce interleukin-2 (IL-2), gamma interferon (INFγ), and lymphotoxin (TNFα), and promote cell-mediated immunity (Fraser et al., 1993). In contrast, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 and promote humoral immunity against extracellular pathogens through activation of B cells and regulation of isotype switching (Mosmann and Coffman, 1989). Several other factors including IL-12, TGFβ, and IL-4 can influence the development of undifferentiated CD4+ T cells towards either the Th1 or Th2 phenotype (Szabo et al., 1993).

The antigen presented by the antigen-presenting cells is recognized in the context of major histocompatibility complex (MHC) class II structures by the T-cell antigen receptor (TCR). Antigen-dependent activation of T cells leads to an immediate early expression and secretion of IL-2, surface expression of the IL-2 receptor, and eventual commitment of T cell to a determined CD4- or CD8-specific function. The TCR–antigen complex activates phospholipase C-γ1 that leads to two separate signals: phosphokinase C (PKC) activation and release of intracellular Ca^{++} stores with a subsequent extracellular Ca^{++} influx. These two signals can be mimicked by treating T cells with agents such as phorbol myristate acetate and ionomycin, a PKC agonist and a Ca^{++} ionophore, respectively.

Regulation of IL-2 Gene Expression and Mechanism of Cyclosporin A (CsA), FK506, and Rapamycin Action

Activation of PKC and Ca^{++} release leads to an immediate early expression of IL-2, correlated with rapid appearance of the NF-AT protein that binds to two different sites in a 300-bp enhancer region, upstream of the IL-2 promoter (Ullman et al., 1990). NF-AT (nuclear factor in activated T cells) is composed of a preexisting cytoplasmic component that translocates upon T-cell activation into the nucleus, designated $NF\text{-}AT_c$ for cytoplasmic (Flanagan et al., 1991) and alternatively,

$NF\text{-}AT_p$ for preexisting (Jain et al., 1992; McCaffrey et al., 1993). The Ca^{++} arm of the T-cell activation pathway activates calcineurin, a calcium-dependent serine/threonine phosphatase that dephosphorylates NF-AT, allowing it to translocate into the nucleus. CsA- or FK506-activated immunophilins block calcineurin function, thereby inhibiting NF-AT translocation to the nucleus and indirectly blocking IL-2 production (for detailed discussion of FK506, CsA and rapamycin mechanism of action, see Quesniaux, 1993). The other arm of the T-cell activation pathway, via PKC, is required for the induction of the ubiquitous AP1 factor that is composed of, at least, Fos and Jun (Jain et al., 1992). Induction of Fos/Jun is essential for the assembly of holo–NF-AT complex. Induction of AP1 is not blocked by CsA or FK506, confirming the two-arm activation strategy of TCR: 1) Ca^{++}-dependent dephosphorylation of NF-AT, and 2) induction of Fos/Jun complex AP1. Rapamycin, on the other hand, does not block IL-2 expression but inhibits the IL-2 proliferative signal in T cells, thus arresting the cells in G-1 phase. FKBP12–rapamycin complex binds to DRR/TOR gene product, a lipid kinase, presumably inhibiting its action and thereby blocking the IL-2 mitogenic response in human cells (Brown et al., 1994).

CsA, FK506, and rapamycin are natural products of microbial origin that have been shown to bind to ubiquitous cytosolic proteins termed immunophilins (Quesniaux, 1993). CsA binds to cyclophilin A, an 18-kDa protein, and FK506 interacts with a 12-kDa immunophilin, named FKBP12. Rapamycin also binds to FKBP12 with 100-fold higher affinity than FK506 (Quesniaux, 1993). These immunosuppressive drugs have various degrees of affinities for a variety of immunophilins. All members of the immunophilin family show a rotamase activity, which enables them to catalyze the *cis-trans* isomerization of peptide bonds involving a prolyl residue and might facilitate protein folding (Schreiber and Crabtree, 1992). Binding of CsA, FK506, and rapamycin to immunophilin generally inhibits the *cis-trans* isomerase activity, but no correlation has emerged between rotamase activity and the immune-suppressive properties of these drug–immunophilin complexes.

Side Effects of the Current Immunosuppressive Therapy

What is the role of immunophilins in the presentation of CsA, FK506, and rapamycin? Immunophilins belong to a relatively large family of proteins and at least 10 members of the family are known. These proteins are also present in abundant quantities in the cell (approximately 10 μM) (Quesniaux, 1993).

It is clear that upon binding to immunophilins these immunosuppressive drugs gain a commanding action that is chaperoned by immunophilins. Given the great abundance of immunophilins in cells, it is remarkable that FK506 and CsA have any therapeutic selectivity. However, both CsA and FK506 have significant side effects and a small therapeutic window (Borel et al., 1989; Bumgardner and Roberts, 1993). Although FK506 is clearly an effective immunosuppressant in organ allografting, the advantage over CsA is at best marginal; the drug is useful in salvaging some patients that are rejecting their transplant with conventional CsA treatment. The major cardiovascular and renal side effects that complicate CsA treatment are no less of a problem with FK506. Gingival hyperplasia, hyperchlolesterolemia, and hirsutism that are associated with CsA therapy are reduced with FK506, but FK506 treatment increases incidence of peripheral neuropathy, gastrointestinal toxicity, and diabetogenicity that are more severe than with CsA (Bumgardner and Roberts, 1993).

The basis of side effects of these drugs is very likely due to the high degree of conservation and numerous interactions with immunophilins. For example, FKBP56, a newly discovered 56-kDa immunophilin, is specifically associated with sex steroid and glucocorticoid receptors (Lebeau et al., 1992; Tai et al., 1992). Treatment of cells with FK506 and rapamycin potentiates the effect of progesterone-mediated transcription (Renoir et al., 1994). In another study where the human progesterone receptor function has been reconstructed in a yeast strain where FKBP12 has been deleted, FK506 dramatically potentiated the effect of progesterone and its analogues (Butt et al., 1994, unpublished results). Because yeast do contain an FKBP56 homologue (Renoir et al., unpublished results) it appears that FK506 can modulate several other transcription functions in cells. The molecular basis of hirsutism observed in female transplant patients treated with CsA or FK506 may be explained by the effects of these drugs on sex steroid receptors.

Targeting NF-AT for Selective Immunosuppressive Therapy

IL-2 plays a commanding role in T-cell activation; thus blocking the IL-2 transcription can disrupt the orchestration of immune response by T cells and lead to immune suppression. Transcrip-

tion factor NF-AT plays a central role in regulation of the IL-2 gene; thus blocking its function will inhibit the T-cell growth as proven by the action FK506 and CsA. It is important to note that in addition to NF-AT, other transcription factors (i.e., NF-κB, AP1, and Oct1) are also involved in the regulation of IL-2 gene expression (Fraser et al., 1993; Rao, 1994). All these factors work in harmony to promote IL-2 expression. However, lessons from biochemical studies and the effect of immunosuppressive therapy suggest that among all the factors, NF-AT plays a key role in IL-2 gene regulation. It also appears that NF-AT may be involved in regulating IL-4, TNFα, and GM-CSF/IL-3 promoters as well (Rao, 1994).

Is it possible to design agents that specifically interfere with NF-AT function? More importantly, realizing that NF-AT may regulate other genes as well, is it possible to identify agents that are specific for NF-AT function as applied to IL-2 gene activation? We believe that combinatorial interactions of NF-AT are unique, and that it may be possible to selectively interfere with IL-2 expression. The unique properties of NF-AT—1) multimeric complex of at least three proteins, 2) heterogeneity in DNA binding sites of target genes, and 3) tissue-specific expression in lymphoid lineages—lends it to varied combinatorial interactions.

As mentioned above, NF-AT is composed of a 120-kDa phosphoprotein and a complex of Fos and Jun, the AP1 subunits. Cloning of the NF-AT_p cDNA from murine (McCaffrey et al., 1993a) and NF-AT_c from human (Northrop et al., 1994) suggest that NF-AT belongs to a small family of proteins. A unifying feature of these proteins is that they contain a Rel homology region of about 270 amino acid residues that presumably binds to DNA (Nolan 1994; Rao et al., personal communication). Although they share 70% homology in the DNA binding domain, outside the Rel domain NF-$AT_{p/c}$ have minimal similarity to each other or to any other protein known thus far in the data base. Amino-terminal splice variants of NF-AT_c have been demonstrated (Northrop et al., 1994), whereas murine NF-AT_p demonstrates a carboxy-terminal alternative splicing pattern (McCaffrey et al. 1993b). The Rel family of proteins NF-κB, p50, p65, and c-Rel show 50–60% homology among their DNA binding domains (Liou and Baltimore, 1993; Nolan, 1994). However, the homology between the DNA binding domain of the Rel family of proteins and NF-$AT_{p/c}$ is 20%, suggesting that the two families are distantly related.

The tissue distribution of NF-AT_c is largely restricted to lymphoid tissues, including T cells, spleen, and thymus. No expression is observed in B-cell lines (Northrop et al., 1994). In contrast, NF-AT_p is expressed in other cells and can be found in brain, heart, and testis (Rao, 1994). Another important distinction is that NF-AT_c is inducible by phorbol myristate acetate and ionomycin in T cells, whereas NF-AT_p is not (Nolan, 1994; Rao, 1994).

Another hallmark of the NF-$AT\gamma_{c/p}$ is their property to interact with the Fos/Jun (AP1) family of proteins. These proteins belong to a large bZIP family of DNA binding proteins, which are characterized by a heptad repeat of leucines (a leucine zipper) that is required for dimerization, and a DNA binding domain (Kerppola and Curran, 1991). The Fos family contains at least four members (cFos, FosB, Fra-1, and Fra-2), whereas the Jun family contains at least three members (cJun, JunB, and JunD). NF-AT binds cooperatively with cFos and cJun to the distal NF-AT site of IL-2 promoter (Jain et al., 1993). cFos and cJun do not bind to the NF-AT site in the absence of NF-AT, suggesting that specificity of binding at the site is conferred by NF-AT. The fully assembled complex of holo-NF-AT may contain Fos-Jun heterodimer or Jun-Jun homodimer (Jain et al., 1993). Detailed studies suggest that Fos and Jun clearly stabilize the interaction of holo-NF-AT with the IL-2 promoter. Thus, combinatorial interactions between bZIP and the Rel family can lead to variety of transcription factor multiprotein complexes, which are open to exciting developments in the field of "transcription factors as drug targets." Given that potential NF-AT binding sites are present at the promoter regions of IL-4, TNFα, and GM-CSF/IL-3 genes, several questions remain. Which members of the NF-AT family interact at different sites of cytokine genes? How do different members of the bZIP family play a role in specificity and selectivity of NF-AT function in regulation of different cytokine promoters?

Tissue-specific expression of NF-AT, heterogeneity in the NF-AT response elements, and multitudes of transcription factor supracomplexes that are possible between bZIP and Rel family will ensure that the field of molecular immunology remains a very rich area in terms of basic research and drug discovery.

CARDIOVASCULAR DISEASES: HIGH-DENSITY LIPOPROTEINS

Overview

In recent international guidelines and recommendations for the prevention of coronary heart

disease (CHD), it was concluded that there is sufficient evidence to support a causal association between plasma high-density lipoprotein (HDL) cholesterol levels and the subsequent development of CHD. Indeed, reduced HDL plasma levels are considered the best single indicator for the risk of coronary heart disease. Conversely, increased HDL plasma levels are associated with protection against CHD and longevity (reviewed in Gordon and Rifkind, 1989; Tall, 1990; Karathanasis, 1992a; Lacko, 1994). Recent work has shown that transgenic mice overexpressing human apolipoprotein AI (apoAI), the major protein constituent of HDL, protects against dietary-induced atherosclerosis (Rubin et al., 1991; Schultz et al., 1993), and injection of apoAI or HDL into rabbits fed with high-cholesterol diets induces regression of preexisting aortic fatty streak lesions (Badimon et al., 1990). Furthermore, overexpression of human apoAI in mice with apolipoprotein E (apoE) deficiency and increased tendency to develop advanced atheromas significantly reduces atherosclerosis susceptibility (Paszty et al., 1994).

Plasma HDL steady-state levels are determined by the rates of HDL genesis and HDL catabolism. The rate of HDL genesis is directly correlated with apoAI and apoAI mRNA synthesis. In mammals, apoAI mRNA is synthesized primarily in liver and intestine, and its expression is regulated by the interplay of multiple transcription factors bound to the apoAI gene regulatory regions. Among them, there is a subgroup of nuclear receptors that includes the retinoic acid receptors (reviewed in Karathanasis, 1992b). Indeed, retinoids increase apoAI gene expression in cultured cynomolgus monkey hepatocytes (Kaptein et al., 1993) and raise apoAI and HDL plasma levels in rabbits (Katocs et al., 1993) and rats (Boehm and Heymman, 1993).

The rate of HDL catabolism is influenced by numerous factors such as hypertriglyceridemia, insulin sensitivity, obesity, lipase activity, and activity of cholesteryl ester transfer protein (CETP). Although it has been recently suggested that the common mechanism of action of these factors is the reduction of HDL size (Brinton et al., 1994), this has been clearly documented only in the case of the action of CETP. It seems that CETP depletes HDL of cholesteryl esters and enriches it with triglycerides (Melchior et al., 1994). These triglycerides are hydrolyzed by lipases, and the core of the HDL particle shrinks into particles with very short life spans (reviewed in Tall, 1993). Indeed, individuals deficient in CETP have greatly elevated plasma HDL levels (Brown et al., 1989; Inazu et al., 1990), and transgenic mice expressing human or monkey CETP have reduced plasma HDL levels (Agellon et al., 1991; Marotti et al., 1993).

This section focuses on the transcriptional mechanisms conferring tissue specificity and regulated expression of the apoAI gene in liver. Our objective is to highlight opportunities for rational development of transcription-based drugs useful for raising HDL plasma levels and atherosclerosis prevention.

Genomic Organization and Tissue-Specific Expression of the apoAI Gene

The genes coding for apoAI and two other apolipoproteins, namely apolipoproteins CIII (apoCIII) and AIV (apoAIV), are physically linked and tandemly organized within an approximately 15-kb DNA segment in the genomes of mammalian (Karathanasis, 1985; Haddad et al., 1986) and avian (Lamon-Fava et al., 1992) species. In mammals, all three of these genes are expressed predominantly in liver and intestine (Zannis et al., 1985; Haddad et al., 1986; Karathanasis et al., 1986). In avian species, in contrast to the mammalian species, the apoAI gene is also expressed in many other tissues in addition to liver and intestine (Lamon-Fava et al., 1992).

Transcriptional Control Regions

Transient transfection analysis of the human apoAI gene using human hepatoblastoma, HepG2 cells (Sastry et al., 1988) and experiments with transgenic mice (Walsh et al., 1989) indicated that a 256-bp DNA region located immediately upstream of the apoAI gene transcription start site (+1) is necessary and sufficient for liver-specific expression. Similar experiments using human intestinal carcinoma Caco-2 cells indicated that sequences extending approximately 2.0 kb to the 5′ direction are also required for intestine-specific expression (Sastry et al., 1988). However, experiments with transgenic mice suggested that sequences 3′ to the apoAI gene, located between the nearby apoCIII and apoAIV genes, are essential for intestine-specific expression (Walsh et al., 1993). More recent work suggested that both apoAI 5′ flanking sequences and sequences between the apoCIII and apoAIV genes are required for high-level expression in both HepG2 and Caco-2 cells (Ginsburg, et al., 1995).

Cis-*Acting Elements*

Systematic deletion mapping analysis of the region conferring liver-specific expression of the

apoAI gene revealed a powerful hepatocyte-specific enhancer located between nucleotides −222 and −110 upstream from the transcription start site (Widom et al., 1991). DNase I protection and electrophoretic mobility shift assays showed that liver and HepG2 cell nuclei contain factors that bind with high affinity and specificity to three sites: sites A (−214 to −192), B (−169 to −146), and C (−134 to −119) within this enhancer (Widom et al., 1991; Ladias and Karathanasis, 1991). Site-directed mutagenesis designed to prevent factor binding to these sites, individually or in different combinations, revealed that simultaneous occupation of all three of them by nuclear factors is essential for maximal enhancer activity in HepG2 cells (Widom et al., 1991). More recent studies showed that although site C contributes to the overall strength of the enhancer, its occupation by nuclear factors is not absolutely essential. Elimination of nuclear factor binding to site C reduces enhancer activity by only 40%. In contrast, elimination of nuclear factor binding to either sites A or B reduces enhancer activity to near background levels (Harnish et al., 1994). Furthermore, these experiments indicated that factor occupation of any of these sites alone (i.e., in the absence of factor binding to the remaining sites) is not sufficient for enhancer activity (Widom et al., 1991; Harnish et al., 1994). This is consistent with earlier observations indicating that none of these sites in isolation can stimulate basal promoters when appropriate constructs are transfected into various cells, including hepatic cells (Widom et al., 1991).

It therefore appears that synergistic interactions between factors bound to sites A and B play a fundamental role in activation of the apoAI enhancer in liver cells. The molecular basis for this synergy is not clear. However, based on the observation that oligonucleotides spanning these sites bind efficiently factors in HepG2 nuclear extracts, it has been suggested that this synergy is unlikely to be due to factor–factor interactions facilitating cooperative binding to DNA (Widom et al., 1992).

Trans-*Acting Factors*

The above observations focused our efforts on the identification and characterization of the transcription factors that bind to sites A and B.

Site A. Screening of several λgt11 libraries with a concatenated site A probe resulted in isolation of the cDNA for ARP-1, a member of the nuclear receptor superfamily (Ladias and Karathanasis, 1991). ARP-1 is an evolutionary relative of Ear-3/COUP-TF (Miyajima et al., 1988; Wang et al., 1989) and Ear-2 (Miyajima et al., 1988), two other members of the nuclear receptor superfamily (recently compiled in Laudet et al., 1992; Amero et al., 1992). ARP-1, Ear-3/COUP-TF, Ear-2, and their heterodimeric versions have been shown to bind with very high affinity to site A (Ladias and Karathanasis, 1991; Ge et al., 1994; Malik and Karathanasis, unpublished). Because ligands for these receptors have not been identified, they are grouped in a subfamily referred to as "orphan receptor superfamily" (Evans, 1988; O'Malley and Conneely, 1992). Subsequent work revealed that site A can also be bound by several other members of the nuclear receptor superfamily. Thus, HNF-4, another orphan receptor (Sladek et al., 1990), and RXRα, a retinoic acid receptor (Mangelsdorf et al., 1990; Rottman et al., 1991) bind to site A and activate nearby basal promoters either constitutively, in the case of HNF-4 (Ge et al., 1994) or in response to retinoids in the case of RXRα (Rottman et al., 1991; Widom et al., 1992; Ge et al., 1994). Furthermore, site A can be bound with high affinity by heterodimers composed of RXRα and the retinoic acid receptors RARα, RARβ, RARγ (Widom et al., 1992; Herman et al., 1992) or heterodimers between RXRα and ARP-1 (Widom et al., 1992) or the peroxisome proliferator-activated receptor (PPAR) (Kliewer et al., 1992). These observations suggested that the apoAI gene is regulated by signaling mechanisms similar to those regulating genes responsive to steroid/thyroid hormones. Moreover, these findings raised the possibility that diverse physiological signals may be transmitted, via site A, to the apoAI enhancer, thus modulating apoAl gene transcriptional activity.

In parallel studies, it was found that some of these nuclear receptors (i.e., ARP-1, Ear-3/COUP-TF, Ear-2, and their heterodimers) repress apoAI enhancer activity (Ladias and Karathanasis, 1991; Widom et al., 1992; Ge et al., 1994; Kilbourne and Karathanasis, unpublished) whereas others (i.e., RXRa and RXRα/RARα or RXRα/RARβ heterodimers) reverse this inhibition in the presence of appropriate ligands (Widom et al., 1992; Ge et al., 1994; Kilbourne and Karathanasis, unpublished). It thus appears that site A functions as a "sensor" whereby the prevailing balance of the intracellular concentration and activation potentials of all of these transcriptional regulators is reported to the apoAI enhancer, which responds accordingly by increasing or decreasing its functional capacity. Clearly, changes of this balance would, ultimately, induce changes

(positive or negative) in the overall rate of apoAI gene transcription.

Site B. As mentioned above, occupation of site B by nuclear factors is essential for transcription activation of the apoAI enhancer in liver cells. Recent evidence indicates that HNF3b, a member of a family of liver-enriched transcription factors related to the *Drosophila* homeotic gene product fork head (Lai et al., 1991, 1993), binds to site B and potently activates nearby basal promoters in hepatic and nonhepatic cells (Harnish et al., 1994). Curiously, the apoAI enhancer that contains site B is not activated by HNF3b in any of these cell types. However, coexpression of both HNF3b and HNF4 potently activates the enhancer in nonhepatic cells while either HNF4 or HNF3b alone do not (Harnish et al., 1994; also see below). This is remarkable, considering that in these cells, expression of the apoAI gene is completely silent.

These observations are consistent with the idea that establishment of the liver phenotype is dependent upon the simultaneous expression of multiple transcription factors acting in specific combinations for each gene (Lai and Darnell, 1991; Xanthopoulos and Mirkovitch, 1993) and provide further support for the notion that synergistic interactions between factors bound to sites A and B play a fundamental role in activation of the apoAI enhancer in liver cells. More importantly, these findings suggest that at least for some promoter contexts, the transcriptional activity of HNF3b is regulated (positively or negatively) by members of the nuclear receptor superfamily. Indeed, the activation of the apoAI enhancer in CV-1 cells (a nonhepatic cell type) by HNF-4 and HNF-3b is abolished by simultaneous expression of ARP-1 or Ear-2 (Harnish and Karathanasis, unpublished). This may have important implications in mammalian development, because both HNF-4 and HNF-3b appear to function in more upstream events leading to the hepatic phenotype (Tronche and Yaniv, 1992; Kuo et al., 1992) and HNF3b has been shown to play an important role in early embryonic development (Weinstein et al., 1994; Ang and Rossant, 1994).

That two prominent developmental factors (i.e., HNF-4 and HNF3b) play a major role in activation of the apoAI gene was unexpected, particularly because apoAI does not seem to play any known role in development. Thus, humans or mice, with their apoAI gene deleted or inactivated, do not present developmental abnormalities (Karathanasis et al., 1987; Li et al., 1993). It is therefore conceivable that, in addition to their roles in development, HNF-4 and HNF-3b are also involved in housekeeping tasks such as the maintenance of liver-specific gene expression in fully developed hepatocytes.

Synergy, Antagonism, and Transcriptional Signaling Cascades

It is clear from the preceding that binding of various members of the nuclear receptor superfamily onto the apoAI enhancer site A and functional interactions between these factors and factors bound to site B (i.e., HNF3b) modulate apoAI gene expression in the liver. A hierarchy of transcriptional signaling events on the apoAI enhancer could be envisaged. First, hepatic cells respond to various extracellular signals by altering the balance of the intracellular abundances and activation (or repression) potentials of nuclear receptors that bind to site A. Second, this information is transmitted to site A by relevant DNA-protein interactions. Then the information is transmitted to site B by synergistic interactions between factors bound to sites A and B. Finally, the transcriptional signal is transmitted to the apoAI basal promoter where it is incorporated, along with other signals, into the basal transcription machinery, thus altering apoAI gene transcription rates. Consistent with this, disturbance of this balance by overexpression of ARP-1, Ear-3/COUP-TF, or Ear-2 results in occupation of site A by these factors, which represses apoAI gene expression (Ladias and Karathanassis, 1991; Widom et al., 1992; Ge et al.; 1994; Kilbourne and Karathanasis, unpublished), presumably because these factors either do not participate in productive interactions with HNF3b at site B, or negatively influence its activity. Interestingly, negative influence of ARP-1 on HNFa activity has been shown to play an important role in apolipoprotein B (apoB) gene regulation in hepatic cells (Paulweber et al., 1993). In an analogous fashion, overexpression of HNF3b in nonhepatic cells does not activate the apoAI enhancer because the prevailing balance of factors that bind to site A favors ARP-1, and Ear-3/COUP-TF or Ear-2 (Harnish et al., 1994; Malik and Karathanasis, unpublished). Not surprisingly, therefore, although overexpression of either HNF-4 or HNF3b alone does not activate the apoAI enhancer in these cells, overexpression of both together does (Harnish et al., 1994). This is presumably due to alteration of the prevailing balance of factors that bind site A in favor of HNF4, which positively influences the activity of HNF3b bound to site B.

Thus, although the exact mechanisms have not yet been worked out, it appears that transcriptional signals arriving at site A in the apoAI enhancer are "processed" by site B before they can ultimately alter apoAI gene transcriptional rates.

Repression-Dependent Switching Between Alternative Transcriptional Activation States

As mentioned above, RXRα and retinoic acid or HNF-4 are potent activators of minimal promoters linked to site A. Similarly, HNF3b activates minimal promoters linked to site B. Therefore, it is surprising that none of these activators alone stimulates the apoAI enhancer, which contains both sites A and B, in hepatic or nonhepatic cells (Widom et al., 1992; Harnish et al., 1994). This suggests that the activities of factors bound to sites A and B are constrained by their natural context within the apoAI enhancer. In nonhepatic cells where the apoAI enhancer is inactive, the constraints on HNF3b activity at site B could be due to the occupation of site A by endogenous ARP-1, Ear-3/COUP-TF, or Ear-2, as discussed above. Similarly, the constraints on RXRα and retinoic acid or HNF-4 at site A could be due to endogenous factors occupying site B or other nearby sites.

In hepatic cells where the enhancer is very active, however, the mechanism for these constraints appears to be more complex. For example, although the enhancer is not activated further by RXRα and retinoic acid, it becomes fully responsive if it is first repressed by ARP-1, Ear 3/COUP-TF, or Ear-2 (Widom et al., 1992; Ge et al., 1994; Kilbourne and Karathanasis, unpublished). This repression-mediated sensitization is not limited to RXRα and retinoic acid because several other activators also overcome ARP-1-mediated repression (Ge et al., 1994). These observations have been interpreted as follows. In liver cells, sites A and B are occupied by transcription factors that are involved in synergistic interactions that maintain apoAI gene expression. Because the abundance and activation potentials of the nuclear receptors that bind to site A are expected to be in constant flux due to their involvement in multiple regulatory cascades, sustained expression of the apoAI gene in response to a specific signal may not be possible. This, however, could be prevented if factors that bind sites A and B participate in sufficiently strong interactions that prohibit their unrestrained replacement by other factors that also bind to these sites. Thus, the ability of exogenous HNF-4, RXRα, and retinoic acid or HNF3b to further activate the apoAI enhancer in hepatic cells would be constrained by virtue of preexisting strong interactions between endogenous factors bound to sites A and B. These interactions, therefore, provide a common mechanistic explanation for both the problem of sustained expression of the apoAI gene in the context of a continuously changing milieu of factors capable of binding to site A, and the inability of various exogenous activators to further activate the apoAI gene enhancer in hepatic cells.

This interpretation, however, poses the following paradox. If these interactions are very strong, then the apoAI gene will be refractory to regulation by factors meant to regulate its expression by binding to site A. We believe that the repression-mediated sensitization of the apoAI enhancer described above resolves this paradox. Thus, repression by ARP-1, Ear-3/COUP-TF, Ear-2, or their heterodimeric versions disrupt these interactions, thereby allowing other activators that bind to site A to establish new productive interactions with factors bound to site B. In this respect, temporally repressed states of transcription could serve as obligatory intermediates in switching expression of the apoAI gene between alternative transcriptional activation states. It is therefore interesting in this context that fluctuations in ARP-1, EAR-3/COUP-TF, and Ear-2 levels in response to retinoic acid treatment of embryonal carcinoma cells modulate Oct4 gene transcription in these cells (Schoorlemer et al., 1994).

We further imagine that this repression–reactivation is a continuous process that allows the apoAI enhancer to constantly "sample" the intracellular environment for changes in the balance of abundance and activation potentials of factors that bind to site A. Thus, according to this model, the apoAI gene site A is targeted by multiple signals and functions as a point of integration of diverse signaling pathways. Clearly, differential sensitivity of different activated states to different repressors and selective responsiveness of the different repressed states to different activators could provide an enormous potential for combinatorial regulation of apoAI gene expression.

Therapeutic Intervention

The synergy between factors bound to sites A and B and the observation that site A can be occupied by many different members of the nuclear receptor superfamily raised the possibility that modification of the intracellular levels or transactivation potentials of these nuclear receptors by

defined chemicals (for example, ligands) may facilitate synergy with factors bound to site B, resulting in upregulation of apoAI gene transcription. For example, as mentioned above, overexpression of HNF-4 and HNF3b in nonhepatic cells activates the apoAI gene. Similarly, overexpression of RXRa and HNF3b in these cells activates the apoAI gene only in the presence of 9-*cis*-retinoic acid, the natural ligand for RXRa (Harnish and Karathanasis, unpublished). We therefore tested the idea that by feeding rabbits with retinoids, it may be possible to change the balance of factors that bind to site A in favor of activated retinoic acid receptors, which could facilitate synergy between sites A and B, resulting in increased transcription of the apoAI gene and increased apoAI and HDL plasma levels. The results of this experiment showed that indeed both apoAI and HDL plasma levels in these animals were significantly elevated in response to retinoic acid feeding (Katocs et al., 1993). These and other similar observations using different experimental animals and various retinoic acid analogues (Boehm and Heyman, 1993) have opened up a potentially fruitful avenue for the development of retinoids as transcription-based drugs useful for raising plasma HDL levels and atherosclerosis prevention. Clearly, the selectivity and efficacy of these compounds will have to be further evaluated.

Future Prospects

The continuously mounting evidence for a causal role of HDL and apoAI in atherosclerosis prevention have led to the establishment of drug discovery programs for raising plasma HDL levels. Basic research investigations on the mechanisms regulating expression of the apoAI gene in liver have begun revealing multiple targets for the development of transcription-based drugs. Retinoids, by activating retinoic acid receptors that bind to the apoAI gene liver-specific enhancer, have emerged as a class of compounds that raise plasma HDL levels in experimental animals. In addition to the retinoids, other ligands or compounds that influence the abundance or activity of various nuclear receptors, or other transregulators that also bind to the apoAI enhancer, may prove to be therapeutically useful. Finally, targeting the entire transcription factor multiprotein complex, which includes transregulators that do not bind to the apoAI enhancer directly, may reveal novel compounds and mechanisms of action. Although it may not be an exaggeration to speculate that chemicals that raise HDL plasma levels will be identified in the near future, their efficacy in atherosclerosis prevention in humans will require long-term clinical studies.

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