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VITAMIN D_3 RECEPTORS: Structure and Function in Transcription

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PERSPECTIVES AND SUMMARY

The vitamin D_3 endocrine system serves principally to coordinate calcium and phosphorus homeostasis through actions on the intestine, kidney, and bone (30, 32, 54, 59). The early discovery that ongoing RNA as well as protein synthesis was required for the above actions prompted the hypothesis that vitamin D_3, like the steroid hormones estrogen, progesterone, and the glucocorticoids, modulates gene expression. The finding that vitamin D_3 is metabolized sequentially in liver and then in kidney to the highly active form 1,25-dihydroxyvitamin D_3 added credence to this hypothesis, as did the observation that 1,25-dihydroxyvitamin D_3 is capable of modulating the expression of specific gene products that play a role in mineral metabolism. The important link between 1,25-dihydroxyvitamin D_3 and its possible action on the genome, however, is derived from the discovery and subsequent characterization of an intracellular protein that selectively binds this active form of vitamin D_3. Ongoing studies by numerous investigators to understand the distribution, structure, and function of the vitamin D_3 receptor have contributed significantly to our current understanding of the mechanism of action of the vitamin D_3 hormone.

The vitamin D_3 receptor has been characterized biochemically from a variety of tissues derived from many animal species (51, 94, 113). The proteins exhibit species-specific molecular weights that range between 48,000 and 60,000 and bind both 1,25-dihydroxyvitamin D_3 and DNA. The ability to bind DNA suggests that the receptor plays a role in modulating gene expression. This property proved to be useful in the isolation of the protein from several tissue sources. In fact the receptor was subsequently utilized successfully in the generation of vitamin D_3 receptor-specific monoclonal antibodies. These immunologic reagents provided the technical methodology whereby complementary DNAs that encoded portions of the structural gene for the chicken vitamin D_3 receptor were first recovered from intestinal cDNA expression libraries. Complete cDNAs for this receptor from rat and human sources have now been identified and their primary structures revealed. The molecular cloning of the vitamin D_3 receptor occurred at the same time that the receptors for the known steroid hormones, as well as receptors for thyroid hormone and the vitamin A derivative retinoic acid, were also identified. Comparison of the primary sequence of the vitamin D_3 receptor with each of these proteins has revealed a similarity in structural organization that suggests that they all belong to a common gene family (38). This family now includes not only receptors for the known steroid, thyroid, vitamin D_3, and retinoic acid hormone ligands but also an equally large group of related proteins for which ligands as well as function have not been ascribed (103).

The purpose of this review is to summarize recent experiments that provide strong support for the original proposal that the active form of vitamin D_3 functions mechanistically as a steroid hormone. We consider very briefly early evidence for this mechanism; we then focus upon more recent experiments that have employed molecular biologic techniques to answer questions about the structure and function of the vitamin D_3 receptor, the possible role of the hormone in receptor activation, and the nature of DNA-binding sites on 1,25-dihydroxyvitamin D_3–responsive genes that mediate receptor function. We conclude this article by discussing recent results suggesting that genetic mutations within the vitamin D_3 receptor chromosomal gene form the underlying molecular basis for the human syndrome of hereditary 1,25-dihydroxyvitamin D_3–resistant rickets.

VITAMIN D_3 HORMONE

Metabolism to the Active Form

Vitamin D_3 is metabolized sequentially in the liver to 25-hydroxyvitamin D_3 and then in the kidney to 1,25-dihydroxyvitamin D_3 (30, 32, 54, 59). The latter form of vitamin D_3 is believed to represent the biologically active metabolite, functioning in the intestine to stimulate calcium and phosphorus absorption, in bone to coordinate the remodelling actions of osteoblasts and osteoclasts, and in the kidney to modulate resorption of minerals. The pivotal role of the renal 25-hydroxy-1α-hydroxylase in vitamin D_3 metabolism is emphasized by the exquisite and complex ionic and hormonal controls placed upon the enzyme's capacity to synthesize 1,25-dihydroxyvitamin D_3. Calcium and phosphate blood levels represent major determinants of 1-hydroxylase activity and therefore of 1,25-dihydroxyvitamin D_3 biosynthesis; they function directly or, in the case of calcium, principally through the modulating actions of parathyroid hormone (12, 46, 63, 142). While both ions are likely to be the most significant positive regulators of 1,25-dihydroxyvitamin D_3 production, additional factors such as estrogen (122, 143), prolactin (137), growth hormone (136), and insulin (132) also play regulatory roles in the metabolite's biosynthesis. The dominant effects of these hormones, interestingly, emerge principally during natural periods of mineral stress that occur during physiologic states characterized by enhanced mineral requirements (11, 120, 123, 137). Significant negative regulation of 1,25-dihydroxyvitamin D_3 production is exerted, as might be expected, by the hormone itself (54). The net result of this complex system is that normal circulating levels of 1,25-dihydroxyvitamin D_3 are precisely controlled and fluctuate only in response to the mineral needs of the organism.

Biologic Actions

The major function of the vitamin D_3 hormone is to coordinate cellular processes in intestine, kidney, and bone that are essential to mineral homeostasis. These events include tissue-specific regulation of calcium-binding proteins such as the intestinal and renal calbindins (24) and a number of bone proteins that include osteocalcin (124), matrix gla protein (44), collagen (130), and alkaline phosphatase (80). The dominant role that 1,25-dihydroxyvitamin D_3 plays in mineral homeostasis is dramatically highlighted by the disease phenotypes observed in human nutritional rickets as well as in other diseases in which vitamin D_3 is either unavailable or improperly metabolized (52). In recent years, however, other actions of 1,25-dihydroxyvitamin D_3 on biologic processes incidental to or unrelated to mineral homeostasis have been discovered. These processes include the modulation by 1,25-dihydroxyvitamin D_3 of the levels of its precursors in skin (37), the biosynthesis of 24,25-dihydroxyvitamin D_3 in the kidney (144), and its degradation in target tissues and cells (51). The hormone is known to modulate the secretion of a number of polypeptide hormones that include not only parathyroid hormone (131) but calcitonin (99), prolactin (150), and insulin (68) as well. Moreover, 1,25-dihydroxyvitamin D_3 has been demonstrated to modulate cellular proliferation and differentiation in hematopoietic cells (1, 7, 81), lymphopoietic cells (74), bone cells (35), and epidermal keratinocytes (61). These effects may be exerted through the control of differentiation-linked proto-oncogenes such as *c-fos* and *c-fms* and replication-linked proto-oncogenes such as *c-myc* and *c-myb* (94). 1,25-Dihydroxyvitamin D_3 is also known to modify B and T lymphocyte activity and therefore the immune response (83), likely through regulation of the level of cytokines such as Il-1, Il-2, Il-3, GM-CSF, γ-interferon, TGF-β, and TNF-α as well as immunoglobulins. These and additional novel biologic phenomena under 1,25-dihydroxyvitamin D_3 control are currently the focus of considerable research effort.

Transcriptional Mechanism of Action

Early studies with inhibitors of RNA and protein synthesis suggested that the actions of vitamin D_3, and subsequently 1,25-dihydroxyvitamin D_3, on intestinal calcium absorption as well as on specific proteins were exerted at the level of the cellular genome (30, 32, 54, 59). These findings prompted the proposal that the vitamin D_3 hormone might function in a manner analogous to that of the steroid hormones exemplified by estrogen (101, 104). The recovery of complementary DNAs for genes that encode such proteins as the calbindins (34, 65), osteocalcin (21), matrix gla protein (44), and parathyroid hormone (57) have enabled direct studies of the effect of 1,25-

dihydroxyvitamin D_3 on specific RNA levels. The results suggest that the vitamin D_3 hormone directly affects the level of these proteins' mRNAs, supporting the original hypothesis that the vitamin D_3 hormone modulates gene expression. Further evidence has been gained through nuclear transcriptional run-on experiments that indicate that the 1,25-dihydroxyvitamin D_3 is effective, at least for a subset of these genes, at the level of mRNA synthesis (94). Nevertheless, despite the extensive number of structural proteins, enzymes, hormones, growth factors, oncogenes, and the biologic responses that appear to be modulated by 1,25-dihydroxyvitamin D_3, evidence that the hormone acts directly on the genome exists for only several of these genes. Definitive evidence for the direct action of 1,25-dihydroxyvitamin D_3 on the expression of a single gene has accrued only for osteocalcin, as is discussed later in this review.

Both early biochemical (16, 55, 145) and later autoradiographic (67, 98, 139, 140, 156) studies have demonstrated that 1,25-dihydroxyvitamin D_3 localizes preferentially to the nuclear chromatin of target cells, a finding that supports the concept of transcriptional action by the vitamin D_3 hormone. The important link between 1,25-dihydroxyvitamin D_3 and its action in the nucleus, however, was only understood after the discovery of the vitamin D_3 receptor. In 1969, Haussler & Norman (56) observed that extraction of the active vitamin D_3 metabolite from intestinal chromatin required buffers containing high salt. The complex was sensitive to protease action and migrated during gel filtration chromatography as a complex of 50,000 to 70,000 daltons. Moreover, the protein was capable of facilitating 1,25-dihydroxyvitamin D_3 association with nuclear chromatin in vitro (15, 17). Together, these observations suggested that the vitamin D_3 hormone was bound to a protein-like macromolecule that exhibited characteristics typical of an intracellular nuclear receptor. Most importantly, this molecule displayed relative affinities for vitamin D_3 metabolites that corresponded to their biological potency in vivo (14). Elucidation of the structure and function of this receptor protein over the past two decades has clearly provided major insights into the mode of action of 1,25-dihydroxyvitamin D_3.

VITAMIN D_3 RECEPTOR

Cellular Distribution

The initial discovery of the vitamin D_3 receptor was accomplished through biochemical examination of avian intestinal mucosa (15, 17, 54). Subsequent studies revealed that the protein is distributed in all avian and mammalian tissues that play recognized roles in mineral regulation. These tissues include intestine, bone, kidney, and parathyroid glands as well as avian shell gland, chorioallantoic membrane, and mammalian placenta (51). Clues to a wider

cellular distribution of the vitamin D_3 receptor, however, emerged as a result of the application of sterol autoradiography. These studies, begun in the intestine (67, 156), were extended to identify vitamin D_3 receptors in the kidney as well as in more novel tissues such as stomach, skin, pancreas, pituitary, and brain (25, 98, 139, 140); all of these receptors were confirmed in analogous tissues through biochemical fractionation procedures. The nature of autoradiographic analysis, however, permitted definitive identification of cell types within tissues that were capable of localizing the 1,25-dihydroxyvitamin D_3 sterol, thereby extending significantly current information regarding specific target cells within complex tissues. Thus, for example, autoradiographic localization revealed the beta cells of the pancreas (25), anterior pituitary thyrotrophs (139), and certain neurons of the brain (140) as likely targets of vitamin D_3 action. Many of these sites such as intestine, kidney, osteoblasts, skin, and stomach were confirmed more recently through the use of immunocytochemical methods employing a monoclonal antibody specific for the vitamin D_3 receptor (10, 26, 100). These studies have revealed likely additional sites of action of the vitamin D_3 hormone that include liver, thyroid, adrenal, esophagus, endometrium, lung epithelium, and lung type II pneumocytes. These studies have shaped the current concept that the vitamin D_3 receptor, like receptors for the glucocorticoids and perhaps retinoic acid, is almost ubiquitously distributed in tissues, although its occurrence is limited to selected cell types within those tissues. This conclusion is also supported by the observation that typical vitamin D_3 receptors can be demonstrated in a wide variety of cultured mammalian cell lines (51). This broad distribution of the vitamin D_3 receptor seems anticipated in view of the extensive biological effects now attributed to 1,25-dihydroxyvitamin D_3.

Biochemical Properties and Subcellular Distribution

Vitamin D_3 receptors are proteolytically sensitive proteins of trace abundance in all target tissues (2, 43, 92, 102). They exhibit species-specific molecular weights that range from approximately 48,000 for the human receptor to approximately 60,000 for the avian receptor (51, 113). The proteins bind 1,25-dihydroxyvitamin D_3 with greater selectivity than they do other vitamin D_3 metabolites; the functional determinants are both 1α- and 25-hydroxyl groups (70, 151, 152). The interaction of the receptor with 1,25-dihydroxyvitamin D_3 is characterized by an equilibrium dissociation constant of approximately 10^{-10} M at 4°C (91), although studies with pure protein have yet to be carried out. This interaction is sensitive to alkylating and sulfhydryl blocking reagents, which suggests the involvement of reactive sulfhydryls in the hormone-binding process (28, 153).

The vitamin D_3 receptor is also capable of interacting in vitro with nuclei and chromatin (15, 17, 56, 111) and immobilized DNA (111, 117). The receptor exhibited a preference for binding to double-stranded rather than single-stranded DNA (111) as well as a preference for adenine:thymine rich base sequences (127). DNA interaction was blocked with the potent DNA-intercalating agent ethidium bromide (118) and, as with 1,25-dihydroxyvitamin D_3 binding, was similarly sensitive to sulfhydryl-blocking reagents such as the organomercurials (110). This biochemical characteristic of binding to DNA appears likely related to the function of the vitamin D_3 receptor in regulating the expression of genes.

The structural relationship between 1,25-dihydroxyvitamin D_3 binding and DNA recognition within the vitamin D_3 receptor was initially identified by Allegretto et al (2, 3). They employed limited trypsin digestion to cleave the chicken receptor protein into two separate domains, a large fragment of 30 to 40 kDa that retained bound 1,25-dihydroxyvitamin D_3 and a smaller fragment that was capable of binding DNA. Immunologic detection of this process was made possible by the monoclonal antibody 9A7, which recognizes an epitope on the vitamin D_3 receptor that functionally inhibits DNA binding (112). The ability to detect the DNA binding domain through immunologic reactivity, and the concomitant loss of immunoreactivity in the hormone-binding fragment, has confirmed the relationship between the 9A7 epitope and a discrete region of the receptor involved in DNA interaction. The ability of carboxypeptidase to modestly reduce the receptor's overall mass while simultaneously causing dissociation of the hormone suggested that the steroid-binding region was carboxy terminal to the DNA-binding domain (4). These data led to the simple concept that the vitamin D_3 receptor is composed of at least two unique functional domains, an amino terminal DNA-binding region and a carboxy terminal 1,25-dihydroxyvitamin D_3–binding domain. Interestingly, distinct abnormalities that affected either 1,25-dihydroxyvitamin D_3 binding or DNA binding have been observed in receptors derived from patients with the hereditary syndrome of 1,25-dihydroxyvitamin D_3–resistant rickets and thus support a similar hypothesis. The molecular cloning of the vitamin D_3 receptor and the subsequent analysis of its structural domains, which are considered later in this review, have confirmed this concept.

Whereas it is generally agreed that the functional site of action of the hormone-activated vitamin D_3 receptor is the nucleus, the location of the receptor prior to formation of its liganded complex has been a matter of controversy. A conclusive answer to this question is important not only because it would establish where initial interactions between the receptor and hormone occur but also because of the suggestion that the vitamin D_3 receptor may retain nongenomic actions in the cytoplasm of the cell. Many of the

biochemical properties of the vitamin D_3 receptor point to the possibility that the hormone-free receptor resides in the nucleus. The protein requires moderately high ionic strength buffers for solubilization during biochemical fractionation (71). Moreover, the vitamin D_3 receptor displays a substantial affinity for nuclei, chromatin, and DNA in the absence of hormone, although that affinity is increased when hormone is added (66, 118). Walters et al (147, 149) carried out an extensive analysis of the behavior of the receptor under a variety of ionic conditions; they subsequently advanced the hypothesis (148) that the vitamin D_3 receptor was predominantly a nuclear protein, regardless of its state of hormone occupancy, much like the thyroid hormone receptor. Studies utilizing immunocytochemical techniques have supported this contention. Thus, both tissues (10, 100) and cultured cells (26) exhibit receptor-specific immunostaining predominantly within the nucleus. More recent studies (8), however, have questioned this result, and suggest that nuclear localization of the receptor in cultured cells may be due to media conditions under which the cells are grown. Clearly, the subcellular compartment to which the unoccupied receptor is targeted following synthesis remains to be unequivocally established. It is likely that resolution of this issue will occur upon demonstration within the receptor of an amino acid sequence that functions as a signal for nuclear localization.

Vitamin D_3 Receptor Regulation

The observation that the expression of the vitamin D_3 receptor is restricted temporally as well as developmentally to certain cell types suggests that cellular mechanisms exist to regulate that expression. This observation is not surprising in view of the fact that the level of receptor represents the major determinant of cellular response to 1,25-dihydroxyvitamin D_3. A number of examples of receptor regulation exist, including modulation of receptor levels by a variety of hormones (22, 39, 108) as well as during chicken embryonic development (97). Perhaps the most interesting example is the correlation between hormone responsiveness and receptor expression in the developing neonatal rat intestine, a model developed extensively by DeLuca and coworkers (19, 31, 49, 62, 86, 109). Rat pups exhibit a limited capacity for intestinal calcium transport during the first several weeks of life. Coincidently, the intestinal epithelial cells of these pups do not express the vitamin D_3 receptor, and the transport system of these animals is refractory to administration of 1,25-dihydroxyvitamin D_3. During the transition to a solid diet at approximately two to three weeks of age, however, the neonatal rat intestine undergoes rapid physiologic maturation (58). Accompanying those changes is the acquisition of capacity to transport calcium, responsivity to 1,25-dihydroxyvitamin D_3, and the appearance of the vitamin D_3 receptor. Expression of the receptor occurs at the level of the mRNA, and glucocorticoids

appear to play an essential role in the induction process (87). This physiologic model provides a striking example of how the appearance of the vitamin D_3 receptor leads to acquired sensitivity to the hormone and to the initiation of biologic response.

1,25-Dihydroxyvitamin D_3 is also a major regulator of the vitamin D_3 receptor, regulation that is likely exerted at a number of levels. Aside from the effect of the hormone in transforming the vitamin D_3 receptor from a transcriptionally inactive to active protein, as is discussed later, 1,25-dihydroxyvitamin D_3 stabilizes the receptor to proteolysis in vitro (88). The hormone is also capable of altering the turnover rate of the receptor in culture cells, although one report suggests that the half-life is increased (27) while another report indicates that no change is evident (107). 1,25-Dihydroxyvitamin D_3 also displays a clear ability to affect the receptor's affinity for the cell nucleus, both in vivo and in vitro, as assessed by the increased ionic strength of buffers that are required to solubilize the occupied form of the protein from nuclear or chromatin fractions (66, 111). Formation of the 1,25-dihydroxyvitamin D_3 receptor complex both in vivo and in vitro also results in a tighter binding to immobilized DNA than is observed with the free receptor alone (66, 118). Interestingly, administration of hormone to chickens in vivo leads to a receptor complex whose affinity for DNA cellulose is somewhat less than that seen for the complex formed in vitro (66). Thus, 1,25-dihydroxyvitamin D_3 directs specific and unique changes to the receptor protein simply as a result of complex formation.

Two additional effects of 1,25-dihydroxyvitamin D_3 are observed in vivo. In a unique study that allowed estimation of receptor-binding activity following induction by a surrogate 1,25-dihydroxyvitamin D_3 (24,25-dihydroxyvitamin D_3), Costa et al (27) first proposed that treatment of cultured mammalian cells with hormonal vitamin D_3 resulted in an upregulation of receptor 1,25-dihydroxyvitamin D_3 binding activity. These studies, as well as additional ones in cell culture (82, 87, 114) demonstrating that this upregulation occurred at the protein and mRNA level, suggested that the vitamin D_3 hormone was capable of autoinducing its own receptor protein, perhaps through transcriptional control. Upregulation of receptor by 1,25-dihydroxyvitamin D_3 was also evident in the rat in vivo (138) and in cultured HL-60 cells (73). In contrast, no increase in receptor was apparent either in chickens in vivo or in organ culture, suggesting that receptor regulation by hormone was species as well as tissue specific. Current efforts focus upon whether the effects of 1,25-dihydroxyvitamin D_3 are exerted at the level of mRNA synthesis or degradation.

1,25-Dihydroxyvitamin D_3 treatment in cultured cells and in organ culture also leads to enhanced levels of phosphorylation of the vitamin D_3 receptor. Pike & Sleator (121) demonstrated that the receptor from cultured mouse

fibroblasts treated with 1,25-dihydroxyvitamin D_3 exhibited a retarded migration during SDS-polyacrylamide gel electrophoresis, which suggests a hormone-dependent modification. Coincubation of the cells with hormone as well as with [^{32}P]orthophosphate, followed by immunoprecipitation, revealed that only the hormone-dependent retarded complex contained an increased phosphate group(s). Phosphorylation appeared to correlate temporally with exposure to the hormone. Subsequent phosphoamino acid analyses revealed that the phosphorylated amino acid species was limited to a serine residue(s) (53). More recent studies by Brown & DeLuca (13) have shown that 1,25-dihydroxyvitamin D_3 treatment of cultured chicken intestine results in a rapid phosphorylation of receptor in that species as well. Despite these experimental results, the role of this receptor modification, apparently induced by 1,25-dihydroxyvitamin D_3, remains elusive. As phosphorylation does not appear to effect either hormone- or DNA-binding, perhaps this modification serves to influence other receptor functions, such as its transactivation capabilities, or provides a signal for receptor degradation. A more complete understanding of the role of phosphorylation will likely come following identification of the specific residue(s) that is involved and the development of appropriate assays that will allow assessment of the effects of this change on receptor properties.

MOLECULAR BIOLOGY OF THE VITAMIN D_3 RECEPTOR

Recombinant Cloning of the Vitamin D_3 Receptor

The development of antibodies directed toward the vitamin D_3 receptor (29, 116, 119), together with the introduction of a new lambda phage cloning vector (155), provided the technical means whereby the receptor's rare structural gene could be recovered from a cDNA library. Thus, the anti-chicken receptor monoclonal antibody 9A7 was utilized by McDonnell et al (89) to screen a randomly primed chicken intestinal cDNA expression library to identify a single viral clone from over 10^7 recombinants. A short cDNA was recovered from the clone and utilized to rescreen additional library recombinants by hybridization, leading to the recovery of several additional clones. DNA sequence analysis of these cDNAs revealed that they each encoded the domain of a protein that exhibited a high degree of homology with that of the *v-erb* A gene product as well as with receptors for the glucocorticoid, progesterone, and estrogen hormones. This domain was strongly related to the multiple DNA binding zinc finger domains of the known transcription factor TFIIIA (93), suggesting a role for this domain in DNA binding and a corresponding role for the protein in transcriptional regulation. Hydrid-selected in vitro translation using the cloned cDNA was

successfully utilized to confirm that the *cDNA* recovered by the 9A7 antibody encoded a portion of the chicken vitamin D_3 receptor. These experimental efforts constitute the molecular cloning of the vitamin D_3 receptor and provide the first direct insight into the relationship between it and other bona fide members of the steroid hormone receptor family of genes (see Figure 1).

Following the recovery of cDNA for the chicken receptor, this probe was utilized by Baker et al (5) to recover cDNA for the human homolog. Partial cDNA clones were isolated by Burmester et al (18) and Pike (115) for the rat receptor, the former through the use of anti-porcine receptor monoclonal antibodies, and the latter through hybridization screening utilizing the human receptor cDNA. The rat cDNA was completed subsequently by Burmester et al (19). The DNA sequence of these clones from different species revealed extensive regions of very high homology, assumed to represent domains

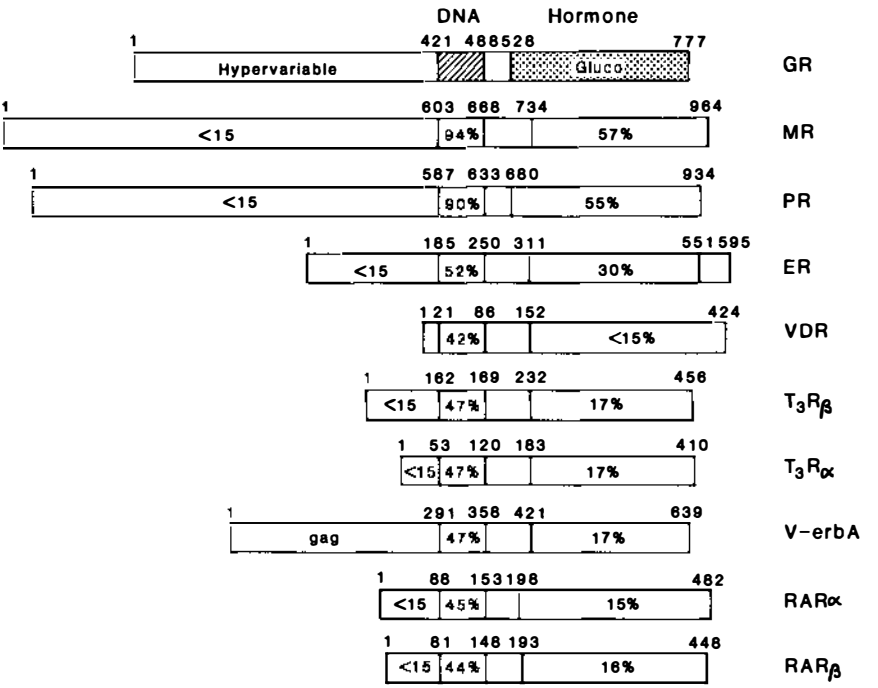

Figure 1 The steroid, thyroid, and retinoic acid receptor superfamily of genes. Schematic comparison of the structural organization of proteins that make up the receptor superfamily. The primary amino acid sequences (numbered above the protein structure) have been aligned on the basis of regions of maximum amino acid sequence identity that are indicated as a percentage relative to the glucocorticoid receptor (GR). DNA- and hormone-binding domains are indicated. Abbreviations of the receptors are GR (glucocorticoid), MR (mineralocorticoid), PR (progesterone), ER (estrogen), VDR (vitamin D_3), T_3R alpha and beta (thyroid hormone), V-erbA (viral erbA protein), and RAR alpha and beta (retinoic acid).

important to function, as well as several smaller regions of lesser homology. The functional importance of these domains is discussed below, but this similarity between the receptors suggests that they have been highly conserved both structurally and functionally through evolution. Unlike the receptors subsequently cloned for the thyroid and the retinoic acid hormones, only a single version of the vitamin D_3 receptor has been identified to date.

Steroid, Thyroid, and Retinoid Receptor Superfamily

In addition to the vitamin D_3 receptor cDNA, representative DNAs for virtually all the known intracellular receptors for the steroid, thyroid, and vitamin A hormones now have been recovered (38, 103). The deduced primary sequences of this class of proteins, together with experiments aimed at dissecting the proteins' domain structure through cDNA mutagenesis, expression, and functional analysis, have led to the realization that all these receptor proteins are structurally as well as functionally related to each other (see Figure 1). These findings suggest that these proteins are a superfamily of receptors whose overall functions are to mediate the genomic actions of their respective cognate ligands.

The use of related receptor cDNAs as probes has led also to the identification of a large number of additional protein members of the steroid receptor family, for which known activating ligands remain uncharacterized (103). While it is possible that these proteins do not require ligands and therefore are constitutively active, the more likely possibility is that they modulate cellular gene expression in response to intracellularly synthesized factors. Perhaps these unknown factors are produced as a result of homeostatic or nutritional cues within the cell. Currently, efforts continue to characterize these especially interesting members of the steroid receptor superfamily as well as to identify new members that may play roles in development, differentiation, and cellular homeostasis.

Structural Organization of the Nuclear Receptors

The deduced primary sequences of receptors for the steroid, thyroid, and retinoic acid hormones reveal similarities in structural organization and two major regions of homology, as indicated in Figure 1 (38). These regions include a strong central or amino terminal core homology as well as a less homologous, but more expansive and complex domain composed of the carboxyl terminal portions of the proteins. The considerable homology exhibited by this family in these regions suggests that they encode domains whose functions are similar within the family.

The protein domain that exhibits the greatest degree of amino acid homology from receptor to receptor represents the DNA recognition structure of the steroid receptor gene family (38). This positionally conserved 60 to 70 amino

acid DNA-binding motif lies at the extreme N-terminus of the vitamin D_3 receptor, although it is more centrally located within receptors for estrogen, progesterone, and the glucocorticoids largely owing to the latter receptors' unique N-terminal extensions. The DNA-binding region is highly basic and is composed of two finger-like projections, each stabilized by a single zinc atom. The metal ions are tetrahedrally coordinated by four positionally conserved cysteine residues located at the base of each loop. Recent studies have suggested that the carboxyl sides of each of the two finger structures more likely take the form of two alpha helixes that interface directly with DNA (50). The DNA binding domain of the vitamin D_3 receptor has been mapped functionally to this conserved zinc finger region, using cDNA deletion mutagenesis coupled to recombinant expression in host monkey kidney fibroblasts. This dual zinc finger motif, as initially exemplified by the *Xenopus* oocyte transcription factor TFIIIA (93), has become the hallmark feature of the steroid receptor family and is diagnostic of a protein with transcriptional modulating activity as well. This DNA binding region in certain of the receptors likely contains additional functions, including weak transactivation capability and the capacity to mediate protein-protein interactions.

The steroid-binding region of the receptor gene family is a highly complex domain that extends from the DNA-binding domain to the carboxy terminus of the protein (38). The precise amino terminal boundary of this domain is uncertain and may vary with each receptor, but it is characterized by a distinct region of intermediate homology within each of the receptors. Mutations introduced into this homology domain obliterate the capacity of the receptors to bind their respective ligands, as do mutations introduced into downstream sites within this extensive domain (47, 72). Deletion of the amino acid sequence N-terminal to this region in the vitamin D_3 receptor, again by deletion analysis, also prevents binding of 1,25-dihydroxyvitamin D_3 (90). Possibly, the N-terminal boundary of the hormone-binding domain of the vitamin D_3 receptor extends further upstream than the boundary for the estrogen or progesterone receptors. Simultaneously, the deletion of approximately 60 amino acids at the carboxy end of the vitamin D_3 receptor also prevents hormone binding. These analyses indicate that the domains forming the hydrophobic hormone-binding pocket of the receptors are highly complex three-dimensional structures. A third region of sequence identity located at the extreme carboxyl terminal and within the steroid receptor family is also present (5, 103); this region exhibits the greatest degree of similarity among receptor subclasses that appear related (e.g. vitamin D_3 and thyroid hormone receptors or progesterone and glucocorticoid receptors). The function of this domain remains to be clarified, although there is some evidence that this region participates in the capacity of the receptors to form either homodimeric (40, 146) or heterodimeric (42, 48) structures.

Role of the Vitamin D_3 Receptor in Transactivation

The fact that the vitamin D_3 receptor is a member of the steroid receptor family of gene transactivators implies that this protein must interact directly with genes known to be modulated by 1,25-dihydroxyvitamin D_3. Definitive evidence for this prediction, however, has emerged only recently for a single gene, that for the bone protein osteocalcin. Previous studies by Baukol & Price (124) suggested that 1,25-dihydroxyvitamin D_3 was capable of modulating the level of this protein in rat osteosarcoma cells in culture. Following the cloning of the complementary DNA for the rat protein (106), these studies were extended to demonstrate that the mRNA levels were also enhanced by the hormone, likely through transcriptional events. The cloning and characterization of the human osteocalcin gene by Celeste et al (21) and subsequently of the rat gene by Yoon et al (154), Demay et al (33), and Lian et al (75) prompted investigation of the effect of the vitamin D_3 hormone at the molecular level.

Several groups initially reported that rat (33, 154) and human (69, 90, 95, 105) osteocalcin gene promoters were inducible directly by 1,25-dihydroxyvitamin D_3 when upstream regions of the genes were inserted into plasmids and utilized to direct transcription of the structural gene for bacterial chloramphenicol acetyltransferase in transfected cells (see Figure 2). These experiments suggested that a discrete DNA sequence that was responsive to the hormone might be present in the 5' region of this gene. Kerner et al (69) used promoter mutagenesis to localize this *cis*-acting vitamin D_3 responsive element (VDRE) within the human gene promoter to a region approximately 500 base pairs upstream of the start site of transcription. Deletion of this short sequence within the osteocalcin promoter led to loss of 1,25-

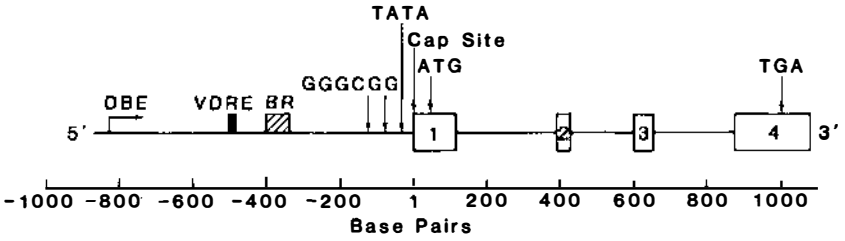

Figure 2 Organization of the human osteocalcin gene. The location of four exons that encode the osteocalcin gene product are boxed and numbered. The cap site and initiation (ATG) and termination (TGA) codons are indicated. The osteocalcin promoter and upstream control regions include the TATA box (TATA), two GC boxes (GGGCGG), basal repressor (BR), vitamin D responsive element (VDRE), and distal basal enhancer (DBE). The nucleotide base scale is indicated below the diagram of the gene.

dihydroxyvitamin D_3 inducibility, whereas the cloning of a synthetic copy of this element into otherwise unresponsive viral promoters, such as for the thymidine kinase gene or the mouse mammary tumor virus long terminal repeat, led to transfer of 1,25-dihydroxyvitamin D_3 response. As shown by Ozono et al (105) more recently, the VDRE is composed of two tandemly repeated hexanucleotide sequences separated by three base pairs. These experiments document the discovery of the first DNA sequence within a promoter capable of mediating direct activation by the vitamin D_3 hormone. Subsequent studies by Demay et al (33) led to the identification of a VDRE located in approximately the same region of the rat osteocalcin gene promoter. The organization of this element is highly conserved relative to the human homolog: 14 of 17 base pairs are identical (105).

The important link between osteocalcin promoter activation by 1,25-dihydroxyvitamin D_3 and the receptor was provided by McDonnell et al (90) and more recently by Ozono et al (105). Both observed that the osteocalcin promoter was uninducible by 1,25-dihydroxyvitamin D_3 when transfected into cultured cells that did not contain the vitamin D_3 receptor. Nevertheless, cotransfection of the osteocalcin promoter together with a plasmid expression vector that directed the synthesis of the vitamin D_3 receptor restored 1,25-dihydroxyvitamin D_3 response. Restoration of response was dependent on receptor concentration (105). These studies clearly establish that the action of 1,25-dihydroxyvitamin D_3 on osteocalcin gene expression, and most likely on other gene promoters, is mediated by the vitamin D_3 receptor. Perhaps as important, these studies functionally confirm that the vitamin D_3 receptor is indeed a ligand-induced transcription factor, thereby cementing the structural relationship that was initially apparent between this receptor and receptors for the steroid, thyroid, and retinoic acid hormone classes. A model for this action on the osteocalcin gene promoter is illustrated in Figure 3.

The identification of a gene responsive to 1,25-dihydroxyvitamin D_3 at the level of transcription has provided an important model system with which to determine the molecular events associated with this activation. Utilizing the transcriptional response assay considered previously, McDonnell et al (90) evaluated a series of mutant vitamin D_3 receptors derived from cDNAs that contained either 3' or 5' deletions of the coding region. The results of this study suggest that transcriptional activation by the receptor requires the presence of an intact DNA-binding domain, but that this domain cannot function independently of the hormone-binding domain. Thus, a transactivation function must reside elsewhere in the receptor molecule, and this function may be under 1,25-dihydroxyvitamin D_3 control. Demay et al (33), Ozono et al (105), Liao et al (76), and Sone et al (134) demonstrated that the vitamin D_3 receptor can indeed interact directly with VDRE sequences in gel retardation or bandshift assays carried out in vitro. Two additional observations become

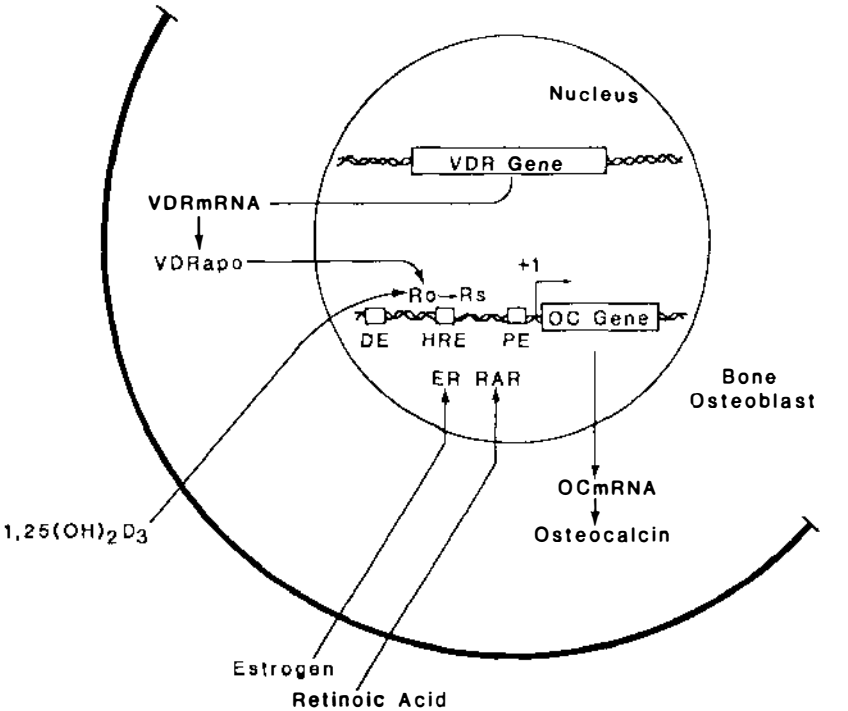

Figure 3 Model for the action of 1,25-dihydroxyvitamin D_3 on osteocalcin gene expression in osteoblasts. The vitamin D_3 receptor (VDR, VDR_{apo}, R_o) is bound to the osteocalcin (OC) promoter (DE, distal element; HRE, vitamin D hormone response element; PE, proximal element), where it is activated by 1,25-dihydroxyvitamin D_3. Transcriptional induction of the osteocalcin gene by the receptor-hormone complex (R_s) leads to the synthesis of new osteocalcin mRNA and protein. Estrogen (ER) and retinoic acid (RAR) receptors may also modulate osteocalcin gene expression.

relevant to this interaction between the receptor and the VDRE. First, as with functional transactivation in cultured cells, the binding of the receptor to the VDRE is dependent upon hormone (76). Thus, while unoccupied vitamin D_3 receptor is unable to form a viable complex with the VDRE during bandshift analysis, the presence of hormone reverses that capacity. Second and equally important is the discovery that the ability of the vitamin D_3 receptor to interact with the VDRE is cooperatively enhanced in the presence of a nuclear protein found in a number of cultured celis (76). Thus, while receptors expressed in mammalian cells containing this nuclear factor bind the VDRE, receptors synthesized through in vitro translation or through synthesis in yeast cells (cells that do not appear to contain the factor) are refractory to binding (134). VDRE binding activity by the vitamin D_3 receptor from these sources can be reconstituted, however, simply by adding mammalian cell extracts to the

incubation mixture. This finding is analogous to that observed for both the thyroid hormone receptor and the retinoic acid receptor (20, 96). Perhaps most interesting is the observation that cooperative interaction between the receptor and the factor that enables the receptor to bind the VDRE occurs weakly in the absence of 1,25-dihydroxyvitamin D_3 (T. Sone et al, unpublished observations). These observations suggest the possibility that a major role of the 1,25-dihydroxyvitamin D_3 hormone is to facilitate receptor-accessory factor dimer formation, which in turn anchors the vitamin D_3 receptor to its cognate response element. Whether the nuclear factor is required for transactivation by the vitamin D receptor, and is thus analogous to certain bridging factors that are currently being evaluated for other basal transactivating proteins (125, 126), remains to be determined.

HUMAN SYNDROME OF HEREDITARY 1,25-DIHYDROXYVITAMIN D_3 RESISTANCE

The human syndrome of hereditary 1,25-dihydroxyvitamin D_3 resistance (vitamin D_3–dependent rickets, type II) is a rare autosomal recessive disease that occurs early in infancy and is characterized by hypocalcemia, secondary hyperparathyroidism, and osteomalacia or rickets (9, 79, 85, 129, 141). These clinical features persist in parallel with elevated levels of circulating 1,25-dihydroxyvitamin D_3, suggesting that they arise as a result of target organ resistance to the action of this hormone. Subtle biochemical and clinical features of the syndrome that vary from patient to patient also suggest the existence of heterogeneity in the nature of the defects that lead to resistance.

Vitamin D_3–Resistant Fibroblast Model

Clinical cases of the syndrome of 1,25-dihydroxyvitamin D_3–resistant rickets were first documented in the late 1970s. However, the discovery that normal skin fibroblasts contain a functional effector system for 1,25-dihydroxyvitamin D_3 provided the key to our eventual understanding of the molecular basis for this disease (36, 41). The subsequent observations that fibroblasts derived from patients resistant to the action of the vitamin D_3 hormone were either qualitatively or quantitatively unresponsive were not surprising (6, 23, 45, 60, 77, 78, 84). Characterization of this protein in defective fibroblasts was initiated rapidly. The cumulative result of these efforts has been the identification of fibroblastic phenotypes in which defective hormone-binding or DNA-binding activities were evident (6, 23, 36, 41, 45, 60, 77, 78, 141). A third defective phenotype was also identified when it was observed that receptors with normal hormone- and DNA-binding functions were incapable of facilitating the nuclear accumulation of 1,25-dihydroxyvitamin D_3 (77,

78, 141). Presently, these three abnormal receptor phenotypes have emerged as potentially responsible for the disease phenotype of rickets (84).

Genetic Lesions in the Vitamin D_3 Receptor Chromosomal Locus

In order to determine the genetic basis for dysfunctional receptors, R. A. Kesterson and J. W. Pike (unpublished results) cloned the human chromosomal gene for the vitamin D_3 receptor and determined its structural organization and nucleotide sequence. Hughes et al (64), Ritchie et al (128), and Sone et al (133) then utilized short oligonucleotide sequences complementary to intron sequences to amplify selectively DNA from each of the coding exons of the vitamin D_3 receptor gene using polymerase chain-reaction techniques. The incorporation of restriction sites at the 5' ends of each synthetic oligonucleotide allowed the subsequent cloning and sequencing of these individually amplified DNAs. In this manner, the presence and nucleotide sequence of each of the exons that encode the human vitamin D_3 receptor were determined rapidly for patients with 1,25-dihydroxyvitamin D_3 resistance.

The search for mutations within the vitamin D_3 receptor gene focussed initially upon analysis of patients whose receptors exhibited defects in ability either to bind 1,25-dihydroxyvitamin D_3 or to recognize DNA. Three interrelated families were evaluated for genetic defects associated with an inability to bind hormonal 1,25-dihydroxyvitamin D_3 (128). DNA amplification and sequence analysis of material from these patients revealed the presence in a single exon of a C to A nucleotide substitution that produced a premature termination codon. Parental DNA was heterozygotic for this mutation, and unaffected siblings were either homozygous or heterozygous for the normal gene. The apparent result of this mutation is the synthesis of a protein truncated at codon 291 within the 1,25-dihydroxyvitamin D_3 binding domain and unable to bind the ligand. Clearly, however, the loss of binding activity in cells can arise from many unrelated defects in the receptor gene, and thus this mutation represents but one example.

The most interesting category of receptor defect in patients with resistance to 1,25-dihydroxyvitamin D_3 leads to altered ability to recognize DNA. As the DNA-binding domain is restricted to two or three exons within the gene, the search for genetic mutations was initiated within those regions using DNA derived from six different families whose receptor proteins exhibited low affinity for immobilized DNA (64, 128; T. Sone et al, unpublished data). Four independent point mutations within these exons that together encode the DNA-binding region of the vitamin D_3 receptor were identified (see Figure 4). These mutations result in aspartic acid or glutamine substitutions at different sites within the dual zinc finger motif that, without exception, result in alterations in the charge of the structure. While it is unclear how these

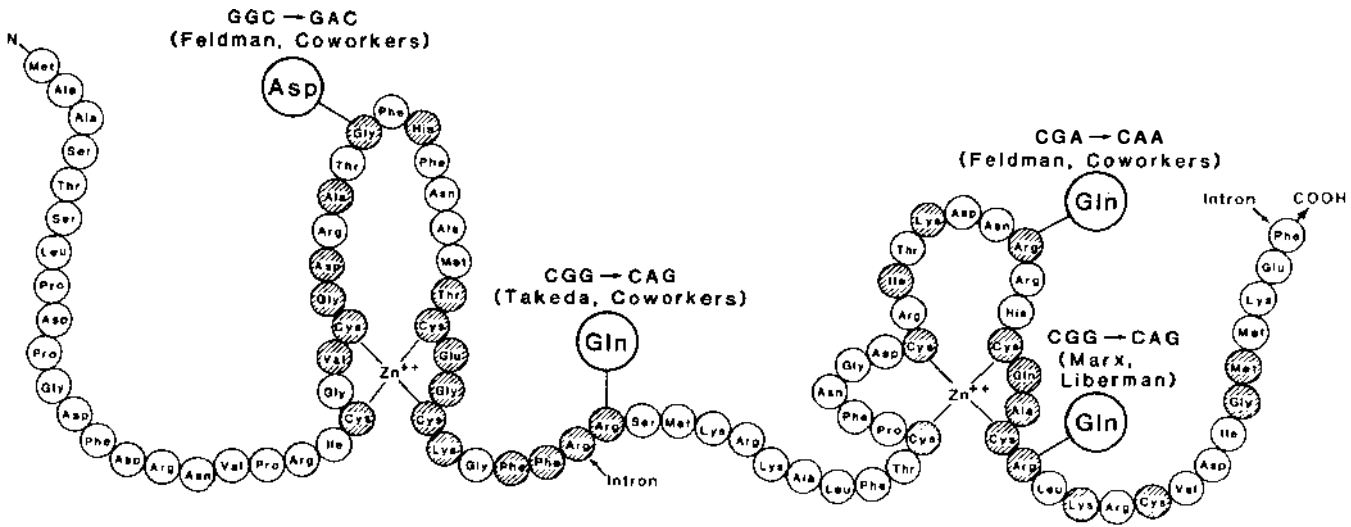

Figure 4 Natural mutations in the DNA-binding domain of the vitamin D_3 receptor identified in chromosomal DNA from patients with hereditary resistance to 1,25-dihydroxyvitamin D_3. The dual DNA-binding zinc finger motif at the amino terminus of the vitamin D receptor is illustrated. Residues that are shaded are conserved within the steroid receptor family. Four amino acid substitutions that have been identified in families with hereditary resistance are indicated. The point mutation in the natural codon that gives rise to the altered residue is summarized over each mutant. Data are derived from References 68, 128, and T. Sone et al, unpublished.

changes affect the DNA-binding activity of the receptor, the preponderance of basic amino acids within this region as well as the conservation of these mutant amino acids within the receptor gene family underscore their importance (38, 103).

Functional Activity of Receptors Derived from Patient Genotypes

To gain additional evidence that the mutations identified within the mutated genes result in aberrant binding functions and inability to modify gene transcription, Hughes et al (64), Sone et al (133, 135), and Ritchie et al (128) utilized the technique of site-directed mutagenesis to introduce each mutation independently into the normal vitamin D_3 receptor cDNA. Analysis of the mutant receptor products synthesized following transfection into host mammalian fibroblasts revealed that each protein exhibited a functional phenotype identical to that in patient fibroblasts. Accordingly, introduction of a termination codon resulted in synthesis of an immunologically detected truncated protein of 32 kilodaltons that was unable to bind 1,25-dihydroxyvitamin D_3 (128). Likewise, the three point mutations introduced individually into the DNA-binding domain of the receptor led to the synthesis of proteins of normal size and ability to bind 1,25-dihydroxyvitamin D_3, but of proteins unable to interact appropriately with either nonspecific DNA (133, 135) or with the osteocalcin VDRE (T. Sone et al, unpublished data). Thus, the defects associated with these mutated receptors correspond identically to those found in their endogenously synthesized counterparts. Perhaps most important, these mutated receptors were also inactive in the transcriptional response assay, outlined above, which was capable of assessing the functional activity of the vitamin D_3 receptor. While the normal receptor was fully capable of activating osteocalcin promoter-mediated transcription in the presence of 1,25-dihydroxyvitamin D_3, mutant forms of the protein did not function, even during receptor overexpression or under conditions of high hormone levels (128, 133, 135). Thus, these defective receptors display not only abnormal binding functions but aberrant transcription-inducing actions as well. These data clearly establish the molecular basis for hormone unresponsiveness in at least a subset of patients with hereditary 1,25-dihydroxyvitamin D_3–resistant rickets. They, in turn, strongly support the essential role of this receptor in mediating the biologic functions of hormonal 1,25-dihydroxyvitamin D_3 that have been described in this chapter.

CONCLUSIONS

The molecular mechanism of action of the vitamin D_3 hormone 1,25-dihydroxyvitamin D_3 parallels that of other steroid hormones. The hormone's action is mediated by the nuclear vitamin D_3 receptor protein that has been

characterized with respect to distribution and regulation as well as physical and functional properties for over two decades. The molecular cloning of the vitamin D_3 receptor in 1987 and 1988, however, proved unequivocally that this protein was related structurally and functionally to the steroid, thyroid, and vitamin A hormone receptor superfamily of genes. Expression of the recombinant vitamin D_3 receptor in heterologous mammalian cells, as well as in mutant forms created by manipulation of its cDNA, has led to substantial clarification of the protein's functional properties and to new and important insights into its mode of action. Simultaneously, the identification of a specific *cis*-acting DNA sequence within the osteocalcin gene, to which the vitamin D_3 receptor binds during gene activation, not only has provided confirmation that 1,25-dihydroxyvitamin D_3 is a steroid hormone but also has provided an important model with which to probe the mechanism of transactivation biochemically. Future challenges to research on the mechanism of action of 1,25-dihydroxyvitamin D_3 remain focussed upon the receptor. Of paramount importance to future efforts is determination of the role that the hormone plays in receptor activation, the mechanisms by which the protein stimulates or represses the activity of promoter elements within responsive genes, and the contribution made by accessory proteins in mediating DNA binding as well as transcriptional activation by the vitamin D_3 receptor. Considering the advances that have made in the past few years, it is likely that at least some of these challenges will be met.

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