We have identified a novel steroid hormone response element in the avian \( \beta_3 \) integrin promoter. This sequence, comprising three hexameric direct repeat half-sites separated by nine and three nucleotides binds vitamin D receptor (VDR)-retinoid X receptor (RXR) and retinoic acid receptor (RAR)-RXR heterodimers. VDR-RXR binds direct repeats separated by three base pairs, and RAR-RXR recognizes half-sites separated by nine bases, whereas the central half-site interacts with both heterodimers. Retinoic acid and 1,25-dihydroxyvitamin D\(_3\) activate both a genomic fragment including the transcriptional start site and an oligonucleotide containing the three repeats, linked to a heterologous promoter. Co-addition of the steroids produces neither synergy nor an additive effect; rather the result equals that for retinoic acid alone. Scatchard analysis demonstrates that RAR-RXR has greater affinity than VDR-RXR for the composite element. Based on these findings we propose a model in which there is specific, polarity-defined binding of VDR-RXR and RAR-RXR to three half-sites, which form two overlapping steroid response elements, with the central half-site common to both. Our results identify a novel mechanism by which one steroid hormone can modulate the activity of a second, by competing for a shared half-site in a composite response element.

Integrins are membrane receptors recognizing specific motifs in matrix proteins and/or cell-associated molecules (1, 2). These heterodimeric molecules play an important role in cell attachment and signal transduction, being responsible for changes in a number of intracellular parameters, all of which share the ability to alter cytoskeletal formation and cell function, (reviewed in Refs. 3–5).

The integrin \( \alpha \beta_3 \) plays a central role in osteoclast function. Proteins such as echistatin or kistrin, containing the \( \alpha \beta_3 \) binding motif, Arg-Gly-Asp (RGD), block bone resorption in vivo and in vitro (6–11), and we have recently identified an RGD peptide mimetic that arrests bone resorption and osteoporosis in the oophorectomized rat. Furthermore, we find a \( \alpha \beta_3 \)-blocking monoclonal antibody blunts the capacity of chicken osteoclasts to resorb bone and attach to osteopontin (13), an RGD-containing bone matrix protein (2, 14).

Little is known concerning regulation of this integrin during formation of osteoclasts from their bone marrow-derived precursors. We demonstrated recently that treatment of avian osteoclast precursors with the osteodestogenic hormone 1,25-dihydroxyvitamin D\(_3\) (D\(_3\)) leads to increased expression of \( \alpha \beta_3 \) on the precursor cells (15).

Steroid hormones modulate gene expression via intracellular receptors binding as dimers to cognate response elements, comprising direct or inverted repeats or palindromes (16–19). In the case of the vitamin D\(_3\) and retinoic acid receptors (VDR and RAR), the transactivating complex also typically contains the retinoid X receptor (RXR) (20, 21), although complexes comprising other combinations of receptors, including VDR-VDR, VDR-RXR, and VDR-thyroid hormone receptor also exist (22–24). Although VDR-RXR usually recognizes direct nucleotide repeats separated by three bases (25–27), there is greater spacer variability for RAR-RXR (19, 26–28). As with other steroids, each VDR-RXR and RAR-RXR complex binds to specific sequences, known as vitamin D and retinoid response elements (VDRE and RARE), respectively.

We have cloned the promoter region of the avian \( \beta_3 \) integrin gene and identified a classical VDRE consisting of two direct repeat half-sites separated by three base pairs (29). We now describe a novel steroid hormone response element in the promoter region of the same gene, comprising three direct repeats separated by three and nine nucleotides, respectively. VDR-RXR binds the first and second half-sites, whereas RAR-RXR associates with the second and third. These data suggest VDR-RXR and RAR-RXR compete for the central half-site of the composite element. In support of this hypothesis, retinoic acid (RA), although itself a weak agonist, completely dampens the more potent transactivation effect of D\(_3\), the active form of vitamin D\(_3\). Thus, we have identified a novel steroid hormone response element in which one steroid hormone modulates the transactivating function of another by competing for a half-site, which is common to two separate response elements.

**MATERIALS AND METHODS**

Generation of Transfection Constructs—The 226 (~185 to +41) and 71-base pair (~185 to ~114) fragments were generated by polymerase chain reaction, using appropriate primers containing the relevant restriction sites and genomic clone 8, known to contain the promoter sequence (29) as a template. The fragments were cloned into pGL2-2

\^1 \text{V. W. Engleman, G. A. Nickols, F. P. Ross, M. A. Horton, S. L. Settle, P. G. Rumincki, and S. L. Teitelbaum, submitted for publication.}

\^2 \text{The abbreviations used are: D}_{31}, 1,25-

\text{dihydroxyvitamin D}_{3}; VDR, vitamin D receptor; RAR, retinoid acid receptor; RXR, retinoid X receptor; RA, retinoid acid; VDRE, vitamin D response element; RARE, retinoid acid response element; EMSA, electrophoretic mobility shift assay; bp, base pair(s); TR, thyroid hormone receptor.}
Identification of a Novel Steroid Response Element

Basic and tkLuc vectors (29), yielding 226Luc and 71tkLuc, respectively. 3300Luc (−3242 to +41) was obtained by NotI digestion of genomic clone 8, leading to isolation of a 2.5-kb base pair fragment that was ligated in the monocyticorientation into the NotI site of a 800Luc construct (−750 to +41), which had been cloned into pGL2-Basic (29). All cloned fragments were sequenced to confirm identity. In selected studies, a series of oligonucleotides, representing the region −160 to −130, containing mutations and/or deletions as indicated in figure legends, were synthesized and cloned into the minimal promoter tkLuc (29).

Transfection Assays—All transfections were carried out in the avian monocytic cell line HD11, using methods described previously (29). Transfected cells were treated with the appropriate steroid(s), namely 1,25(OH)2D3 (10−8 M) and or retinoic acid (10−7 M) or vehicle for 48 h, at which time cells were lysed, and aliquots of lysate were assayed for luciferase and β-galactosidase. A single result represents the mean of triplicate transfections. The entire set of experiments was repeated four times, and interassay variation averaged 25%.

Electrophoretic Mobility Shift Assays—Nucleotides used as probes for EMSA were either the previously described −185 to −114 fragment (obtained by polymerase chain reaction) or synthetic sequences covering all or portions of the region −162 to −129 region in the avian β3 promoter. Other synthetic oligomers used were those encoding the published VDRE of the human osteocalcin promoter (30) and the glucocorticoid response element from the human growth hormone promoter (31). The proteins used in these studies were baculovirus-expressed VDR, RAR, and RXR. The antibody 9A7, to the avian VDR was provided by Dr. Wesley Pike (Ligand Pharmaceuticals, La Jolla, CA), whereas Dr. Pierre Chambon (Strasbourg, France) kindly donated antibodies 8J (to the RARα) and RX-1D12 (to RXR). EMSA was performed by incubating 32P-labeled probe with or without various combinations of VDR, RAR, and RXR. In selected tubes a designated excess (5-100-fold) of an unlabeled oligonucleotide was added as competitor. For studies aimed at establishing the identity of proteins in a given complex, some incubations also contained the monoclonal antibody 9A7, 8J, or RX-1D12. Following an incubation period of 20 min at 22 °C, samples were separated in a 4% sequencing gel, and the gel was dried and exposed to film (Kodak X-Omat).

Methylation Interference Studies—The oligonucleotide −185 to −114, generated by polymerase chain reaction as described above, was labeled on either the top or bottom strand and partially methylated using published methods (32). VDR-RXR bound complex (B) or free DNA (F) were isolated, cleaved with piperidine, and analyzed in a 9% sequencing gel.

Scatchard Analysis—The affinity of the VDR-RXR and RAR-RXR complexes for their respective response elements were determined essentially as described (24). In brief, fixed amounts of each heterodimer were incubated with varying amounts of 32P-end-labeled DR3 (for VDR-RXR) or DR9 (for RAR-RXR). The resulting protein-DNA complexes were separated in a 4% sequencing gel, and the bands representing free and bound ligand were identified followed drying and autoradiography. Quantitation was established by excising each band, which was counted in a β scintillation counter. Standard Scatchard plots of the data allowed determination of the appropriate Kd value.

RESULTS AND DISCUSSION

A reporter plasmid, 226 Luc, containing a short fragment (−185 to +41) of the avian β3 promoter, but not the previously reported (29) classical VDRE (−785 to −770), was transfected into the avian monocytic line HD11. The transfected cells were treated, in turn, with combinations of D3 and RA. Transcription is stimulated 4-fold by D3 treatment, whereas RA is a weaker agonist, transactivating the construct approximately 1.5-fold. Co-treatment with D3 and RA yields a result indistinguishable from that of RA alone (Fig. 1).

The 226-bp fragment contains, at −151 to −131, two direct repeats of a consensus half-site (GGGGCA) separated by nine bases (DR9), similar to a previously described VDRE (33). When avian cells transfected with a reporter plasmid containing a 71-bp oligonucleotide (−185 to −114) including the DR9 sequence linked to a tk promoter were treated with combinations of D3 and RA, the results parallel those obtained with the 226-bp genomic fragment (Fig. 1). Thus, this smaller fragment retains the capacity to respond transcriptionally to both D3 and RA. Most importantly, transfections with a reporter construct containing a large fragment of the promoter, including the transcriptional start site (−3242 to +41), mirror the results observed with the 226-bp genomic fragment (Fig. 1). Thus, the native gene and smaller constructs are regulated in a like manner.

To identify the RARE and VDRE within 71tkLuc, we performed EMSA using a 32P-labeled oligonucleotide (−185 to −114). Both VDR-RXR and RAR-RXR associate with this 71-bp fragment (Fig. 2A). When a shorter probe, comprising only DR9 and immediate flanking bases (−152 to −129) was used, RAR-RXR formed a retarded band (Fig. 2B). In contrast, RXR and VDR at a concentration twice that which shifts the 71-bp probe (see Fig. 2A) fails to retard DR9, suggesting that an unrecognized VDRE is present elsewhere in the 71-bp fragment. To identify this VDRE, we turned to methylation interference studies. As seen in Fig. 3, we identified, at −161 to −146, a VDR-RXR-recognizing sequence, DR3, composed of two direct repeats including the upstream GGGGA half-site of DR9 separated by three bases from a more 5′ hexanucleotide (GGAGCT). Evidence that this sequence represents a functional VDRE is provided by transfection studies, the results of which are summarized below (see Fig. 5). Thus, we have identified a novel response element in the proximal region of the avian β3 integrin promoter containing three direct repeats (3DR) separated by three and nine nucleotides (see Fig. 3 for details).

Specificity of VDR and RAR binding to the composite steroid response element was confirmed in oligonucleotide competition studies and by the use of antibodies directed to individual protein components of the putative heterodimers. Thus, oligonucleotides coding for a consensus VDRE (Fig. 4B) and DR9 (Fig. 4C) but not a glucocorticoid response element displace VDR-RXR and RAR-RXR heterodimers, respectively, in a concentration-dependent manner.

Consistent with our model, antibodies to VDR and RAR prevent formation of VDR-RXR and RAR-RXR complexes, respectively, whereas anti-RXR antibody almost completely ablates binding of either heterodimer to 3DR (Fig. 4A). The presence of a faint supershifted band in the experiment using the RXR antibody (Fig. 4A, lane 10), while anomalous, is not without precedent. Similar findings in which an antibody that normally completely blocks formation of a retarded oligonucleotide-
Steroid receptor complex leads to formation of a faint supershifted band has been reported for both 9A7, the anti-VDR reagent used in this study (34) and a monoclonal antibody against the estrogen receptor (35).

The recognition of DR3 and DR9 by VDR-RXR and RAR-RXR, respectively (Fig. 4), predicts that D3 and RA exert their transcriptional effect via these individual nucleotide repeats. To determine if such is the case, we deleted the first or third half-sites of the composite response element or mutated the central half-site (common to both the putative VDRE and RARE) and measured changes in the capacity of the mutant to support transactivation of a reporter gene. As seen in Fig. 5, the intact element is activated by either D3 or RA. As predicted by our model, deletion of either the most 5' or 3' half-sites results in loss of response to D3 and RA, respectively. Mutation of the central half-site, in either the intact or deleted elements, leads to loss of activation by both hormones.

These data establish that the β3 integrin promoter contains a novel steroid response element composed of three direct repeats separated by three and nine nucleotides, which acts as a combined VDRE and RARE. The fact that both VDR-RXR and RAR-RXR recognize the middle half-site suggests that at any time, only one heterodimer complex can bind 3DR. If such is the case, exposure of cells transfected with 3DRluc to either D3 or RA will result in occupancy of DR3 and DR9 by VDR-RXR and RAR-RXR, respectively, leading in each case to transactivation. The RA-like stimulation of luciferase activity following co-addition of D3 and RA (Fig. 1) can be explained if the affinity of RAR-RXR for the response element is greater than that of VDR-RXR. To test this hypothesis we performed Scatchard analysis, using as ligand the two half-sites derived from the composite element, using as competitors various concentrations of 32P-labeled DR3 and DR9 oligomers. Again consistent with our hypothesis, the affinity of the RAR-RXR complex for its binding site is significantly higher than that of VDR-RXR and its corresponding response element (Fig. 6). Thus, the affinity of RAR-RXR for its composite element is greater than that of VDR-RXR.

In summary, we find the β3 integrin promoter contains 3DR, a novel, composite steroid response element composed of three direct repeats separated by three and nine nucleotides, which acts as a combined VDRE and RARE. The fact that both VDR-RXR and RAR-RXR recognize the middle half-site suggests that at any time, only one heterodimer complex can bind 3DR. If such is the case, exposure of cells transfected with 3DRluc to either D3 or RA will result in occupancy of DR3 and DR9 by VDR-RXR and RAR-RXR, respectively, leading in each case to transactivation. The RA-like stimulation of luciferase activity following co-addition of D3 and RA (Fig. 1) can be explained if the affinity of RAR-RXR for the response element is greater than that of VDR-RXR. To test this hypothesis we performed Scatchard analysis, using as ligand the two half-sites derived from the composite element, using as competitors various concentrations of 32P-labeled DR3 and DR9 oligomers. Again consistent with our hypothesis, the affinity of the RAR-RXR complex for its binding site is significantly higher than that of VDR-RXR and its corresponding response element (Fig. 6). Thus, the affinity of RAR-RXR for its composite element is greater than that of VDR-RXR.

In summary, we find the β3 integrin promoter contains at least two elements responding to D3, one of which also contains a functional RARE. Because RA blunts D3-mediated transcription of a reporter construct (226luc) containing only 3DR, as well as one (3300luc) comprising both VDREs (the classical one at −785 to −770) and the composite element (3DR at −160 to −131), it appears that the composite element dominates in the context of the native promoter. The intricacy of this process underscores the complex mechanism by which D3 and RA interact to modulate osteoclast function (36–38). In particular, given the central role played by the integrin αvβ3 in osteoclast function, our findings are consistent with the recently reported

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**Fig. 2.** The 71-bp oligonucleotide contains both vitamin D and retinoic acid response elements. A, both VDR-RXR and RAR-RXR bind the 71-bp DNA fragment in EMSA analysis. EMSA was performed with a 32P-labeled 71-bp oligonucleotide (−185 to −114), incubated alone (lane 1) or with baculovirus-expressed VDR (lanes 2, 5, and 6) or RAR (lanes 4, 5, and 6) with RXR present in lanes 2–6. B, the RAR-RXR complex, but not VDR-RXR, binds to DR9. A synthetic oligonucleotide (−152GGGGGACGGGAGGGGACT−129) containing two half-sites (in bold) separated by nine nucleotides, was used as a probe in EMSA assays, which also included RXR and either RAR or VDR (1× or 2×). The presence of excess VDR fails to generate a VDR-RXR heterodimer, indicating that this complex does not form on DR9.

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**Fig. 3.** Identification of a binding site for VDR-RXR in the 71-bp fragment. Methylation interference analysis on the 71-bp fragment (−185 to −114) using a VDR-RXR complex for protection reveals a third half-site (GGAGCT) upstream of those previously identified. The nucleotide sequence of the top and bottom strands is illustrated on the right and left, respectively. The guanine residues whose methylation interferes with VDR-RXR-DNA complex formation are indicated by dots. The same G residues are shown with dots in the corresponding sequence (lower panel). Arrows mark the direct repeats identified by a combination of this procedure, plus the earlier results.
antagonistic effect of RA on D$_3$-induced differentiation in avianosteoclast precursors. Although RA has been shown to inhibit gene transcription via various promoter elements (39–43), our findings demonstrate a unique mechanism by which this steroid hormone modulates transcriptional activity by sharing a common binding site, namely the RXR element. One consequence of this sharing is that it allows prediction of binding polarity of the heterodimeric complexes to the composite element. Thus, the overall polarity, moving along the composite element in the 5'-to-3' direction, is predicted to be VDR-RXR-RAR. This model is of considerable interest in the light of recent reports demonstrating that different RXR-containing heterodimers bind with defined polarity to DNA. Thus, several reports indicate that for TR-RXR and RAR-RXR complexes the RXR component is always 5'-of its partner (23, 44–47). These findings are consistent with the specificity of RXR binding in the composite element.

**Fig. 5.** Selective deletions and mutations of half-sites in 3DR, DR3, and DR9 blunt D$_3$- or RA-induced transcription. Synthetic sequences containing 3DR, DR3, DR9, or various mutations and deletions were ligated into a minimal tkLuc promoter and used in transfection assays, as described under "Methods and Materials." Deletion of the upstream and downstream half-sites blocks D$_3$- and RA-stimulated transcription, respectively. Mutation of the central half-site eliminates both events.

**Fig. 6.** RAR-RXR binds to 3DR with higher affinity than does VDR-RXR. A series of EMSA experiments were performed with fixed amounts of VDR-RXR and RAR-RXR complexes, using either the DR3 or DR9 oligonucleotides as probes, respectively. Following incubation and electrophoresis, free and bound bands were identified by autoradiography, excised, and counted. The $K_d$ for each heterodimer-response element pair was determined by Scatchard plots of the data.
Identification of a Novel Steroid Response Element

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