Reconstitution of the Vitamin D-Responsive Osteocalcin Transcription Unit in *Saccharomyces cerevisiae*

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The human osteocalcin gene is regulated in mammalian osteoblasts by 1,25(OH)₂D₃-dependent and -independent mechanisms. The sequences responsible for this activity have been mapped to within the -1339 region of the gene. We show here that this enhancer region functions analogously in *Saccharomyces cerevisiae* cells engineered to produce active 1,25(OH)₂D₃ receptor. When fused to the proximal promoter elements of the yeast iso-1-cytochrome *c* gene, the enhancer demonstrated substantial promoter activity. This activity was elevated further by 1,25(OH)₂D₃ when the reporter constructs were assayed in cells containing the 1,25(OH)₂D₃ receptor. This system affords a model for 1,25(OH)₂D₃ action and represents a simple assay system that will enable definition of the important *cis*-acting regulatory sequences within the osteocalcin gene and identification of their cognate transcription factors.

The major noncollagenous protein of bone is osteocalcin, a 49-amino-acid protein (12). Initial studies of the expression of this protein (15) and subsequently of its mRNA (28) have shown it to be restricted to osteoblast or osteoblastlike cells. In cells where it is produced, it has been demonstrated that the expression of this protein is biphasic (21). In the absence of hormonal stimulation, there is a high rate of basal activity, whereas administration of physiological concentrations of 1,25(OH)₂D₃ elicits a further sixfold induction. This hormone responsiveness has been demonstrated to be a result of *de novo* transcription events associated with administration of 1,25(OH)₂D₃, and it is implied that these responses are receptor mediated and represent classic steroid hormone action (L. P. Pan and P. Price, *J. Bone Min. Res.* 1[Suppl. 1]:20, 1986). The gene for this protein has been isolated, and the promoter has been defined (5, 8, 32). We have shown that the sequences responsible for basal and hormone-inducible activities of the human gene reside within a region downstream of -1339, which we have defined as the enhancer region of this gene (25). This sequence confers hormone responsiveness on a heterologous promoter in any receptor-containing cells but exhibits basal activity only in homologous ROS 17/2.8 osteosarcoma cells. This pattern of activity suggests the involvement of tissue-specific factors that either repress basal activity in heterologous cells or promote activity in homologous cells. We are interested in defining further the regulation of this gene, with the aim of developing it as a model system to study 1,25(OH)₂D₃ action. Two approaches have been taken. The first is the classic route of using deletion analysis to define precisely the sequence responsible for 1,25(OH)₂D₃ responsiveness (S. Kerner, R. S. Scott, and J. W. Pike, *Proc. Natl. Acad. Sci. USA*, in press); the second approach is to study the response element in its natural gene and investigate the interaction of this enhancer with other modulators within the same sequence. To this end, we have adopted a novel approach to studying the regulation of this enhancer in the yeast *Saccharomyces cerevisiae*.

It previously had been shown that yeast enhancers can

confer regulation on a heterologous gene in mammalian cells (14, 29) and that steroid-responsive elements can confer hormone responsiveness on heterologous genes in yeasts when transformed into yeast cells expressing recombinant mammalian steroid receptor (19, 25). We therefore reasoned that in yeast cells we may be able to study both components of osteocalcin expression separately and in the absence of tissue-specific modulators. This paper describes the reconstitution of a hormone-responsive transcription unit in yeast cells and the use of a novel gene fusion technology to engineer yeast cells that produce active 1,25(OH)₂D₃ vitamin D receptor (VDR).

MATERIALS AND METHODS

Biochemicals. Restriction enzymes were purchased from Promega Biotec (Madison, Wis.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and Amersham Corp. (Arlington Heights, Ill.). T4 polynucleotide kinase and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc. (Bethesda, Md.). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tritiated 1,25(OH)₂D₃ (93 Ci/mmol) was purchased from Amersham, and radioinert 1,25(OH)₂D₃ was purchased from Duphar (Da Weesp, The Netherlands).

Strains. The yeast strain F762 (MATa *trp1 ura3-52 CUP1'*) (4b) was used throughout.

Construction of reporter plasmids. The reporter plasmids YRPV3a and YRPV3b were constructed by inserting the 1-kilobase-pair (kbp) *Bam*HI-*Sac*I fragment of the human osteocalcin gene (5) into a standard shuttle vector after addition of a *Bam*HI linker at the 3' end. The fragment was excised with *Xho*I and *Sall* and cloned into the unique *Xho*I site of PLGSD5G (11). YRPV4 contains this same fragment cloned into the *Xho*I site of plasmid pC2 (a gift from Denis Thiele). YRpV6 and YRpV7 were constructed by synthesizing DNA corresponding to the -538 to -469 and -442 to -368 regions of the human osteocalcin gene. These were made with *Xho*I cohesive ends and cloned into the unique *Xho*I site of pC2. YRpP3 was constructed by cloning the -132 to -750 region of the chicken ovalbumin gene, which was treated with DNA polymerase (Klenow fragment), into

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FIG. 1. Yeast expression vectors. The full-length cDNA for the $1,25(\text{OH})_2\text{D}_3$ receptor was inserted so as to produce a fusion protein with ubiquitin (A) or as an unfused molecule (B). UB, The 76-amino-acid ubiquitin molecule; *CUP1*, the yeast metallothionein promoter.

a Klenow-filled *Xho*I site of pC2. The reporter pCL1 was a gift from Denis Thiele. Plasmids were transformed into F762 or into F762 containing the receptor expression plasmid and screened for the presence of the appropriate auxotrophic markers.

Construction of expression vectors. YE pV1, a high-copy-number yeast expression vector, was constructed as follows. A synthetic linker with the sequence

AATTGCTTAAGACTAAGAGGTGGTGAATTCGGTAC
CGAATTCTGATTCTCCACCACCTTAAGC.

which encoded the carboxyl six amino acids of ubiquitin and contained an internal *Eco*RI site, was synthesized. This linker was cloned into the *Eco*RI-*Kpn*I site of pGEM4. The resulting plasmid, pG42, was digested with *Eco*RI, and the 2.0-kbp *Eco*RI-*Eco*RI fragment of pHVDR13 (2) was inserted into that site. In the correct orientation, this gave plasmid BCpV1, containing an in-frame fusion of ubiquitin to the receptor. After amplification, the *Afl*III-*Kpn*I fragment was excised and subcloned into the corresponding sites of YE p46 (4b). The resulting plasmid was called YE pV1 (Fig. 1A). A second expression construct, YE pV3, was constructed by digesting YE p136-GOAT (a gift from Jeff Stadel) with *Nco*I-*Pvu*II and filling in the 5' overhang with T4 DNA polymerase. This was ligated to the 2.0-kbp *Eco*RI-*Eco*RI fragment of pHVDR13, which previously had been blunt ended with DNA polymerase. A plasmid containing the insert in the correct orientation was called YE pV3 (Fig. 1B). These plasmids were amplified, purified, and used to transform *S. cerevisiae* by using standard protocols (27). Transformants were selected by tryptophan auxotrophy.

Production of recombinant receptor. Cells containing the receptor expression plasmids were grown overnight in minimal medium containing 2% glucose. The next day, the cells were diluted 10-fold and allowed to grow to an optical density at 600 nm of 1.0. Production of the receptor was initiated by the addition of 100 μM copper sulfate. The cells were allowed to grow for an additional 2 h, harvested by centrifugation, and washed twice with ice-cold TK0 (10 mM Tris hydrochloride [pH 7.5], 5 mM dithiothreitol). The final pellet was suspended in 450 μl of TK0, and the cells were disrupted by vortexing three times for 1 min in a microfuge tube containing one-third volume of glass beads (0.5 mm; A. R. Thomas Scientific). A fourth vortexing was done after the addition of 50 μl of 3 M KCl to ensure extraction of the receptor from the nucleus. The cellular debris was removed by centrifugation at $12,000 \times g$ for 10 min. The supernatant was recovered, and the total protein was estimated by using

a protein quantitation system (Bio-Rad Laboratories, Richmond, Calif.). The cytosols were analyzed immediately.

Western blotting (immunoblotting). Proteins from recombinant cells were analyzed by Western blotting as described previously (17) after resolution on a 10% sodium dodecyl sulfate-polyacrylamide gel as described by Porzio and Pearson (24).

Hormone-binding assays. Hormone-binding assays were performed by using 0.1 to 1% (vol/vol) yeast cytosol. Single-point assays were done by incubating 0.1-ml samples (1 to 10 μg of protein) with 1 nM radiolabeled $1,25(\text{OH})_2\text{D}_3$ with or without a 100-fold molar excess of radioinert hormone. After a 2-h incubation at 4°C , specific binding was determined by the hydroxyapatite binding assay as described previously (30). Saturation analysis was carried out analogously, using increasing concentrations of labeled ligand and overnight incubation.

DNA-cellulose chromatography. Cytosol from recombinant cells was prepared as described above and labeled for 2 h with 2 nM ^3H -labeled $1,25(\text{OH})_2\text{D}_3$. The salt concentration was lowered by dilution, and the protein fraction was loaded onto a 5-ml calf thymus DNA-cellulose column. After extensive washing, the specifically bound proteins were eluted by using a linear gradient of KCl. The protocol is described in more detail elsewhere (22).

Assay of promoter activity. Cells containing the reporter plasmids were grown overnight in minimal medium supplemented with 2% glucose. The cells were lysed with glass beads, and the cytosol was clarified by centrifugation ($12,000 \times g$). Samples (1 to 10 μg) of protein were assayed for β -galactosidase activity as described by Miller (20); activity was expressed as units per milligram of protein.

RESULTS

Production of mammalian VDR receptor in yeast cells. It has been demonstrated that recombinant human estrogen receptor produced in yeast cells binds hormone and is capable of directing hormone-dependent activation of genes containing estrogen response elements (19). The reconstitution of a series of eucaryotic regulatory systems in yeast cells suggests that it may be feasible to produce VDR receptor in yeast cells and use this to study regulation of the $1,25(\text{OH})_2\text{D}_3$ -responsive genes. Two types of expression vectors were constructed for this purpose (Fig. 1). The first was a high-copy-number vector (YE pV3) that used the copper-responsive yeast metallothionein (*CUP1*) promoter to drive the synthesis of receptor mRNA when the cDNA for the human VDR was inserted. Initiation in this vector was from the natural AUG of the receptor. The second type of vector, using the same promoter, consisted of a fusion of the cDNA for the receptor to the carboxyl terminus of a synthetic cassette-adapted ubiquitin molecule (46). In this case, initiation was from the ubiquitin AUG, producing a fusion protein. The advantage of this system is that a host processing enzyme removes the fused ubiquitin soon after translation, thus leaving a natural receptor molecule (1, 46). With many other proteins, this system has been shown to enhance the level of production by increasing stability of the proteins and solubility and also shown to protect the molecule from endogenous proteolysis (4a). Cytosol from transformants containing these vectors was analyzed initially by Western blot (Fig. 2A). In this case, the two vector systems directed approximately the same amount of synthesis. However, it is obvious that the receptor produced as a fusion protein (Fig. 2A, lanes 1 to 4) was of better quality than that produced in

FIG. 2. Production of recombinant $1,25(\text{OH})_2\text{D}_3$ receptor in yeast cells. *S. cerevisiae* F762 was transformed with YEpV1 and YEpV3, and receptor synthesis was induced by the addition of $100 \mu\text{M}$ CuSO_4 . Cytosols were analyzed by Western blotting. Lanes contained $10 \mu\text{g}$ (lanes 1 and 3) and $1 \mu\text{g}$ (lanes 2 and 4) of total protein extracted from two independent clones transformed with YEpV1 and $10 \mu\text{g}$ (lanes 5 and 7) and $1 \mu\text{g}$ (lanes 6 and 8) of protein from separate transformations with YEpV3. (B) Inducibility of the receptor quantitated by immunoblotting. Cytosols from uninduced cells and from cells treated with 5 and $100 \mu\text{M}$ CuSO_4 were analyzed.

the unfused state (Fig. 2A, lanes 5 to 8); the latter was extensively degraded, presumably because of proteolysis. On the basis of comparisons with the immunoreactive signal with a known amount of chicken VDR (data not shown), we estimate that these strains are producing the receptor equivalent to approximately 0.2% of the total soluble protein, or about 150,000 molecules per cell. This represents a level 50 to 100 higher than that found in receptor-rich tissues such as intestine (7, 18). A minor immunoreactive band was detected above the receptor in cytosols from YEpV1-transformed cells. We believe that this resulted from reaction with some uncleaved fusion protein and not from a modification by the host because the band was not apparent in cytosol from cells transformed with YEpV3 (Fig. 2, lanes 5 to 8).

The *CUP1* promoter is tightly regulated by copper ions (4), allowing the controlled expression of receptor in yeast cells. This allows study of subtle mutations of the receptor that may demonstrate an apparent normal phenotype in an overexpressed state. To demonstrate the inducibility of the system, cells were grown in the absence of copper and in the presence of 5 and $100 \mu\text{M}$ copper sulfate. After induction, cytosols were prepared and analyzed by immunoblot. Uninduced cells contained no immunoreactive proteins, as anticipated, whereas receptor was detectable after addition of 5 or $100 \mu\text{M}$ copper sulfate (Fig. 2B).

All subsequent analysis of the receptor was done by using the expression vector YEpV1. The hormone-binding properties of the recombinant molecule are shown in Fig. 3. In cytosol from cells transformed with the receptor expression plasmid, a high-affinity saturable binding site was detected (Fig. 3A) that was not present in wild-type cells. The affinity of this receptor was found to be $4 \times 10^{-10} \text{ M}$ when the data were transformed by the method of Scatchard (Fig. 3B). This

FIG. 3. Analysis of the functional properties of the recombinant receptor. Cytosol containing the recombinant receptor was analyzed by saturation (A) and Scatchard analysis (B). Symbols: ●, total binding; ○, specific binding; □, nonspecific binding. No specific binding of $1,25(\text{OH})_2\text{D}_3$ was observed in cytosols from nontransformed cells. (C) DNA-binding properties of the receptor analyzed by calf thymus DNA-cellulose chromatography. Symbols: ○, elution profile of the tritiated hormone-receptor complex; —, KCl gradient used to elute the bound receptor.

affinity was similar to that observed by others for the wild-type mammalian receptor and similar to our current estimates of the affinity of the mammalian receptor (2). It was estimated that between 40 and 100% of the immunoreactive receptor was able to bind hormone. The discrepancy may be the result of a dynamic process within the cell that is responsible for activation or deactivation of the receptor or of error resulting from the variations inherent in immunoblotting.

The DNA-binding properties of the receptor were examined by using calf thymus DNA-cellulose chromatography, a technique that has been shown to be a good assay of an intact DNA-binding activity (13). Cytosol was labeled with tritiated $1,25(\text{OH})_2\text{D}_3$ and loaded onto a DNA-cellulose column. After extensive washing with low-salt buffer, the bound proteins were eluted with a linear gradient of KCl. The profile (Fig. 3C) demonstrates that the peak of radioactivity eluted at 0.22 M KCl, consistent with results for the wild-type receptor (2).

Transcriptional activity of the recombinant receptor. To test the transcriptional activity of the vitamin D receptor produced in yeast cells, a series of reporter plasmids was

FIG. 4. Transcriptional activation of the VDR in yeast cells. Derivations of YRpV6 and YRpV7 from pC2 are described in Materials and Methods. Only the relevant parts of the plasmids are shown. The *CYC1* sequences are represented by a solid line, with the site of insertion of the oligonucleotides indicated (*XhoI*). pCL1 contains a copper-responsive UAS. Recombinant yeast cells were grown overnight in minimal media supplemented with 2% glucose, with the treatments indicated: +H, addition of 1 μ M 1,25(OH) $_2$ D $_3$; +Cu, addition of 100 μ M copper sulfate. Values are averages and variations from three independent assays. β -Galactosidase activities are expressed as Miller units per milligram of protein.

developed (Fig. 4). Initial experiments were done with constructs derived from the reporter plasmid pC2. This vector contains the yeast iso-1-cytochrome *c* proximal promoter elements (to -250 bp) fused to the *Escherichia coli* β -galactosidase structural gene. The *CYC* upstream activating sequence (UAS) and sequences responsible for reducing the activity of the *CYC* promoter have been removed (10, 11). This particular *lacZ*-*CYC* fusion was chosen because its behavior in yeasts has been well characterized (31) and its responsiveness to mammalian enhancers has been described (19, 25).

A region of the human osteocalcin gene that contains a sequence (VDRE; -538 to -469) conferring 1,25(OH) $_2$ D $_3$ responsiveness on heterologous genes (Kerner et al., in press) was synthesized and inserted into the unique *XhoI* site of pC2 (YRpV7). A similar construct, YRpV6, was created by using a synthetic piece of DNA (VDRE^M) corresponding to the -442 to -368 region of the osteocalcin gene, which does not contain a VDRE, to serve as a negative control. These constructs were transformed into yeast cells containing the receptor expression plasmid, and cotransformants were selected by tryptophan and uracil auxotrophy. These transformants were grown in the presence of 1 μ M 1,25(OH) $_2$ D $_3$ and 100 μ M copper sulfate, and cytosols were analyzed for β -galactosidase activity (Fig. 4). The basal activity of the enhancerless *CYC* promoter (pC2) was independent of the copper or hormone concentration. On the other hand, YRpV7 was shown to substantially activate transcription of the reporter in a copper- and hormone-dependent manner. In this experiment, a sevenfold induction was observed. This responsiveness was specific for constructs containing a VDRE, as the promoter of YRpV6 was unresponsive under identical conditions. The basal rate of transcription of YRpV7 was higher than that of the wild-type vector or YRpV6. The same pattern was observed when this element was analyzed in mammalian cells (Kerner et al., in press), which suggests that this 60-bp fragment may be recognized by other transcription factors. These results also suggest that YRpV7 may be partially activated in a hormone-independent manner (discussed below).

The system responded specifically to 1,25(OH) $_2$ D $_3$, since high concentrations of estradiol or progesterone (1 μ M) fail to activate transcription (data not shown). The high concentration of 1,25(OH) $_2$ D $_3$ required for half-maximal induction (5×10^{-8} M) (data not shown) was 2 orders of magnitude greater than the affinity of the receptor. This anomaly may be due to the impermeability of yeast to the hormone or to

metabolism of the ligand. Steroids have been shown to be extensively modified by yeasts (33). These concentrations of hormone are similar to that required to activate the estrogen receptor in yeast cells (19).

The transcriptional activity of a fully activated *CUP1* promoter contained within plasmid pCL1 was assayed in the same background for comparative purposes. This construction gave approximately 35,000 U of activity, suggesting that, by comparison, the VDRE is not a strong enhancer. This finding is not peculiar to the yeast system but reflects observations made previously in cell culture (Kerner et al., in press).

Regulation of the osteocalcin enhancer. Having demonstrated that the VDR was functional in yeast and was produced in a regulatable manner, we wished to reconstitute a 1,25(OH) $_2$ D $_3$ -responsive transcription unit in yeast cells, primarily to develop a system amenable to genetic dissection of the mechanisms of osteocalcin regulation. Previously, the distal regulatory elements of the osteocalcin gene were shown to be contained within a 1,000-bp segment (-344 to -1339) of the 5'-flanking region. When this fragment was introduced as a single copy into the pC2 reporter (creating YRpV4) and transformed into cells containing the receptor expression plasmids, it demonstrated a high basal activity (4,000 U) that was substantially elevated upon addition of receptor and hormone to the system (Fig. 5). Assay of a fragment containing a similar region of the chicken ovalbumin gene (YRpP3) did not demonstrate either basal or inducible activity. Clearly, this osteocalcin DNA contains sequences that interact with yeast transcription factors to produce a constitutive promoter activity. This activity was independent of receptor plasmid, since it was also observed in cells transformed solely with the reporter plasmid (data not shown). We are in the process of using this system to characterize *in vivo*-generated mutants, with the hope of identifying some of the components of this transcription unit.

We were also interested in altering the basal rate of transcription of the reporter in order to increase the magnitude of the receptor-dependent response. We have shown in a similar system used to reconstitute a progesterone-responsive transcription unit that this can be accomplished by inserting a *GAL* UAS into the construct (P. Mak, D. P. McDonnell, T. R. Butt, N. L. Weigel, and B. W. O'Malley, manuscript in preparation), which has the effect of suppressing genes when the cells are grown in glucose. To test this idea, we constructed similar reporters by insertion of the

FIG. 5. Functioning of the enhancer region of the human osteocalcin gene in yeast cells. Derivations of YRpV3a and YRpV3b from pLGSD5 and YRpV4 and YRpP3 from pC2 are described in Materials and Methods. Only the relevant portions of the plasmids are shown. The *CYC1* sequences are indicated by a solid line, with the site of insertion of the osteocalcin sequences at -250 indicated (*XhoI*). OS1, The -344 to -1339 upstream enhancer region of the human osteocalcin gene; OV1, the -132 to -750 region of the chicken ovalbumin gene; UASG, the galactose-responsive UAS; +H and +Cu, addition of $1 \mu\text{M}$ $1,25(\text{OH})_2\text{D}_3$ and $100 \mu\text{M}$ copper sulfate, respectively, to the growing cells. β -Galactosidase activities are expressed as Miller units per milligram of protein. Values are averages and variations from three independent assays.

enhancer as a single or double copy into a unique *XhoI* site of pLGSD5 (11). In addition to containing the *CYC1* promoter elements, this plasmid has a 310-bp piece of DNA that contains the *GAL* UAS. The activity of a single copy of the enhancer in this reporter (YRpV3a) was approximately 25% that observed in YRpV4. However, it was induced 13-fold by the addition of copper and hormone. Interestingly, when two copies of the enhancer were introduced (YRpV3b), the basal activity increased; this finding indicates that the elements responsible for regulation of the basal transcription activity act synergistically, whereas the addition of a second VDRE apparently does not affect the inducibility of the promoter.

Optimization of the system. The increase in transcriptional activities of reporters containing either a synthetic VDRE (Fig. 4) or the osteocalcin enhancer (Fig. 5) were shown to be a sum of hormone-dependent and hormone-independent activation. We are interested in the mechanisms of the hormone-independent activation. Initially, the vitamin D-responsive reporters were introduced into F762 and examined in the absence of an expression plasmid (data not shown). The basal activities of these reporters were the same in both the presence and absence of copper, suggesting that the hormone-independent activation is not a result of the addition of copper but is receptor dependent. It was demonstrated (Fig. 2B) that the amount of receptor produced was proportional to the copper concentration. It was therefore of interest to determine whether hormone-dependent and -independent activation could be separated by altering the receptor (copper) concentration. For this analysis, cells containing the receptor expression plasmid and YRpV7 were grown in the presence or absence of hormone and with a gradient of copper (Fig. 6). The results showed that hormone-dependent activation was half-maximal at $10 \mu\text{M}$ CuSO_4 , whereas there was minimal activation at this point in the absence of hormone. It therefore appeared that the hormone-independent activation was a result of overexpression of the receptor.

DISCUSSION

The recent demonstration that yeast enhancers and enhancer-binding proteins function in mammalian cells (14,

29), insects (9), and plants (16) and the subsequent demonstration that steroid response elements are operative in yeasts (19, 25) suggest that the basic mechanisms of transcription and its regulation are well conserved. This view is further substantiated by the apparent structural similarity of mammalian and yeast RNA polymerase II (26) and the functional similarity between the yeast and mammalian TATA-binding proteins (3, 6). On this basis, a $1,25(\text{OH})_2\text{D}_3$ -responsive transcription unit that enabled studies of the regulation of the mammalian osteocalcin promoter and provided a system for subsequent genetic analysis was developed in yeast cells.

Our studies have used a novel approach to engineer yeast cells that produces active VDR. The receptor cDNA, when fused to the cDNA of the highly conserved ubiquitin molecule, resulted in the production of a fusion protein. The

FIG. 6. Hormone-dependent and -independent activation of transcription. Cells transformed with YEpV1 and YRpV7 were grown in minimal medium supplemented with 2% glucose with (●) or without (○) the addition of $1,25(\text{OH})_2\text{D}_3$ and increasing concentrations of copper sulfate. After a 24-h incubation, the cells were harvested and β -galactosidase activities were measured. Data are expressed as Miller units per milligram of protein.

fusion is processed soon after translation by the host processing enzyme. This strategy did not enhance the absolute amount of protein synthesized, as it has in other cases, but it significantly increased the amount of intact receptor produced (Fig. 2A). Possible explanations for the protective effect of the ubiquitin are that (i) it protects receptor degradation by translocation to a different cellular compartment or aids the natural folding process or (ii) the host ubiquitin-processing enzyme complex protects the receptor by excluding cellular proteases (4a). This strategy is also being used in our laboratory to produce biologically active human estrogen and chicken progesterone receptors in yeasts.

The expression system was used to reconstruct a steroid-responsive transcription unit in yeast cells. Sequences containing a VDRE were fused to the proximal promoter elements of the enhancerless iso-1-cytochrome *c*- β -galactosidase reporter. This conferred 1,25(OH) $_2$ D $_3$ responsiveness on the heterologous promoter. The VDRE apparently behaves in yeast cells much as has been observed in mammalian cells (Kerner et al., in press).

The primary goal of this project was to produce a 1,25(OH) $_2$ D $_3$ -responsive osteocalcin transcription unit in yeast cells to facilitate a genetic dissection of the important regulatory sequences involved in regulation of the gene. The enhancer region (-344 to -1339) of the human osteocalcin gene has been shown to be responsible for tissue-specific regulation of this gene, and it contains the sequences required for 1,25(OH) $_2$ D $_3$ responsiveness (23). When assayed in the yeast system described here, the osteocalcin enhancer substantially increased the transcriptional activity of the hybrid promoter. This activity was further induced by the addition of hormone-activated receptor to the system. The behavior of this gene, a high constitutive activity that was further induced by VDR, is analogous to the pattern of expression noticed in homologous osteoblast cells (21). These data suggest either that the enhancer contains a sequence recognized fortuitously by a yeast transcription factor which acts positively or that the absence of a mammalian tissue-specific repressor allows constitutive expression. These alternatives are now under investigation, with the aim of using this system to identify elements responsible for the repression of this gene in all but mammalian osteoblast cells.

Use of the copper-responsive metallothionein promoter has decided advantages over use of constitutive promoters because the amount of receptor produced can be controlled. In our study, this system allowed us to underexpress the steroid receptor in situations in which overexpression obviously had led to hormone-independent activation of the gene. We are not sure of the mechanism of this hormone-independent activation. One possibility is that a minor proteolytic receptor fragment is sufficiently active at high concentrations to activate the target gene, although we cannot detect any substantial proteolysis by immunoblot. Alternatively, it may be that when the receptor is greatly overexpressed, a small proportion of the receptor population is in an active conformation and does not require hormone. This latter possibility would suggest that the role of the hormone is to act as a catalyst in an equilibrium reaction involving conversion of inactive receptor to an active form.

The production of active VDR in yeast cells has allowed us to demonstrate directly that osteocalcin gene expression is regulated by VDR upon activation by the steroid. An interesting observation was that the induction of expression of the reporters was independent of the basal rate of transcription. This finding is analogous to what has been ob-

served for regulation of the gene in mammalian cells and illustrates that this gene is controlled by two independent mechanisms (8, 23, 32).

The development of this 1,25(OH) $_2$ D $_3$ -responsive transcription unit and subsequent demonstration that regulation of the transgene was similar to that of the cellular gene in osteoblast cells suggest that this may be a system useful for defining various types of *cis*-acting regulatory regions within genes. In particular, our results illustrate the potential of yeast systems for studies of mammalian genes whose expression is normally restricted to their homologous tissues.

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LITERATURE CITED

1. Bachmair, A., D. Finley, and A. Varshavsky. 1986. *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234**:179-186.
2. Baker, A. R., D. P. McDonnell, M. R. Hughes, T. M. Crisp, D. J. Manglesdorf, M. R. Haussler, J. W. Pike, J. Shine, and B. W. O'Malley. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA* **85**:3294-3298.
3. Buratowski, S., S. Hahn, P. Sharp, and L. Guarente. 1988. Function of a yeast TATA element-binding protein in a mammalian transcription system. *Nature (London)* **334**:37-42.
4. Butt, T. R., and D. J. Ecker. 1987. Yeast metallothionein and applications in biotechnology. *Microbiol. Rev.* **51**:351-364.
- 4a. Butt, T. R., S. Jonnalagadda, B. P. Monia, E. J. Sternberg, J. A. Marsh, J. M. Stadel, D. J. Ecker, and T. Crooke. 1989. Ubiquitin fusion augments the yield of cloned gene products in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**:2540-2544.
- 4b. Butt, T. R., M. I. Khan, J. Marsh, D. J. Ecker, and S. T. Crooke. 1988. Ubiquitin-metallothionein fusion protein expression in yeast. *J. Biol. Chem.* **263**:16364-16371.
5. Celeste, A. J., V. Rosen, J. L. Bueker, R. Kriz, E. A. Wang, and J. M. Wozney. 1986. Isolation of the human gene for bone Gla protein utilizing mouse and rat cDNA clones. *EMBO J.* **5**:1885-1890.
6. Chodosh, L. A., J. Olesen, S. Hahn, A. S. Baldwin, L. Guarente, and P. A. Sharp. 1988. A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable. *Cell* **53**:25-35.
7. Dame, M. C., E. A. Pierce, J. M. Prah, C. E. Hayes, and H. F. DeLuca. 1986. Monoclonal antibodies to the porcine intestinal receptor for 1,25 dihydroxyvitamin D $_3$: interaction with distinct receptor domains. *Biochemistry* **25**:4523-4534.
8. Demay, M. B., D. A. Roth, and H. M. Kronenberg. 1989. Regions of the rat osteocalcin gene which mediate the effect of 1,25 dihydroxyvitamin D $_3$ on gene transcription. *J. Biol. Chem.* **264**:2279-2282.
9. Fisher, J. A., E. Giniger, T. Maniatis, and M. Ptashne. 1988. GAL 4 activates transcription in drosophila. *Nature (London)* **332**:853-856.
10. Guarente, L. 1983. Yeast promoters and lacZ fusions. *Methods Enzymol.* **101**:181-191.
11. Guarente, L., R. R. Yocum, and P. Gifford. 1982. A GAL 10-CYC1 hybrid yeast promoter identifies the GAL 4 regulatory region as an upstream site. *Proc. Natl. Acad. Sci. USA* **79**:7410-7414.
12. Haushka, P. V., J. B. Lian, and P. M. Gallop. 1978. Carboxylated calcium-binding proteins and vitamin K. *N. Engl. J. Med.* **302**:1460-1466.
13. Hirst, M. A., H. I. Hochman, and D. Feldman. 1985. Vitamin D resistant alopecia: a kindred with normal 1,25 (OH) $_2$ D $_3$ binding

- but decreased receptor affinity for deoxyribonucleic acid. *J. Clin. Endocrinol. Metab.* **60**:490–495.
14. **Kakidani, H., and M. Ptashne.** 1988. Gal4 activates gene expression in mammalian cells. *Cell* **52**:161–167.
 15. **Lian, J. B., M. Coutts, and E. Canalis.** 1985. Studies of hormonal regulation of osteocalcin synthesis in cultured fetal rat calvariae. *J. Biol. Chem.* **260**:8706–8710.
 16. **Ma, J., E. Przibilla, J. Hu, L. W. Bogorad, and M. Ptashne.** 1988. Yeast activators stimulate plant gene expression. *Nature (London)* **334**:633–636.
 17. **Manglesdorf, D. J., J. W. Pike, and M. R. Haussler.** 1987. Avian and mammalian receptors for 1,25 (OH)₂D₃: *in vitro* translation to characterize size and hormone dependent regulation. *Proc. Natl. Acad. Sci. USA* **84**:354–358.
 18. **McDonnell, D. P., D. J. Manglesdorf, J. W. Pike, M. R. Haussler, and B. W. O'Malley.** 1987. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science* **235**:1214–1217.
 19. **Metzger, D., J. H. White, and P. Chambon.** 1988. The human oestrogen receptor functions in yeast. *Nature (London)* **334**:31–36.
 20. **Miller, J. H.** 1972. Assays for β -galactosidase, p. 352–355. *In* J. H. Miller (ed.), *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 21. **Pan, L. C., and P. Price.** 1984. The effect of transcriptional inhibitors on the bone Y-carboxyglutamic acid protein response to 1,25(OH)₂D₃ in osteosarcoma cells. *J. Biol. Chem.* **259**:5844–5847.
 22. **Pike, J. W.** 1981. Evidence for a reactive sulfhydryl in the DNA binding domain of the 1,25 (OH)₂D₃ receptor. *Biochem. Biophys. Res. Commun.* **100**:1713–1719.
 23. **Pike, J. W., D. P. McDonnell, R. A. Scott, S. Kerner, R. A. Kesterson, and B. W. O'Malley.** 1988. The vitamin D₃ receptor and the chromosomal gene, p. 147–159. *In* J. Carlstedt-Duke, S. Erickson, and J.-A. Gustafsson (ed.), *The steroid/thyroid receptor family and gene regulation*. Birkhauser-Verlag, Basel.
 24. **Porzio, M. A., and A. M. Pearson.** 1977. Improved resolution of Myo-fibrillar proteins with sodium dodecyl sulphate polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **490**:27–34.
 25. **Schena, M., and K. R. Yamamoto.** 1988. Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science* **241**:965–967.
 26. **Sentanac, A.** 1985. Eucaryotic RNA polymerases. *Crit. Rev. Biochem.* **18**:31–91.
 27. **Sherman, F., G. R. Fink, and G. Hicks.** 1982. *Methods in yeast genetics: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 28. **Theofan, G., and P. A. Price.** 1988. Inhibition of 1,25-dihydroxyvitamin D₃ stimulated BGP mRNA synthesis in ROS 17/2 cells after prolonged treatment with 1,25 dihydroxyvitamin D₃, p. 381–382. *In* A. G. Norman, K. Schaefer, H.-G. Grigoleit, and D. von-Herrath (ed.), *Vitamin D: molecular, cellular and clinical endocrinology*. Walter de Gruyter, New York.
 29. **Webster, N., J. R. Jin, S. Green, M. Hollis, and P. Chambon.** 1988. The yeast UASg is a transcriptional enhancer in human HeLa cells in the presence of the Gal4 trans-activator. *Cell* **52**:169–178.
 30. **Weckslar, W. R., and A. W. Norman.** 1979. An hydroxylapatite batch assay for the quantitation of 1 α -25-dihydroxyvitamin D₃ receptor complexes. *Anal. Biochem.* **92**:314–323.
 31. **West, R. W., R. R. Yocum, and M. Ptashne.** 1984. *Saccharomyces cerevisiae* GAL1-GAL10 divergent promoter region: location and function of the upstream activating sequence UASg. *Mol. Cell. Biol.* **4**:2467–2478.
 32. **Yoon, K., S. J. C. Rutledge, F. Buenaga, and G. A. Rodan.** 1988. Characterization of the rat osteocalcin gene: stimulation of promoter activity by 1,25 dihydroxyvitamin D₃. *Biochemistry* **27**:8521–8526.
 33. **Zedan, H. H., O. M. El-Tayeb, and M. Abdel-Aziz.** 1976. Microbiological transformation of steroids. *Planta Med.* **30**:360–368.