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Transcriptional Regulation of the *Bmp2* Gene

RETINOIC ACID INDUCTION IN F9 EMBRYONAL CARCINOMA CELLS AND *SACCHAROMYCES CEREVISIAE**

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***Bmp2*, a highly conserved member of the transforming growth factor-β gene family, is crucial for normal development. Retinoic acid, combined with cAMP analogs, sharply induces the *Bmp2* mRNA during the differentiation of F9 embryonal carcinoma cells into parietal endoderm. Retinoic acid (RA) also induces the *Bmp2* gene in chick limb buds. Since normal *Bmp2* expression may require an endogenous retinoid signal and aberrant *Bmp2* expression may cause some aspects of RA-induced teratogenesis, we studied the mechanism underlying the induction of *Bmp2*. Measurements of the *Bmp2* mRNA half-life and nuclear run-on assays indicated that RA stimulated the transcription rate of the *Bmp2* gene. The results of ribonuclease protection and primer extension assays indicated that *Bmp2* transcription started 2,127 nucleotides upstream of the translation start site in F9 cells. To identify genetic elements controlling this transcription rate increase, upstream and downstream genomic sequences flanking the *Bmp2* gene were screened using chloramphenicol acetyltransferase reporter genes in F9 cells and β-galactosidase reporter genes in *Saccharomyces cerevisiae* that were cotransformed with retinoic acid receptor and retinoid X receptor expression plasmids. RA-dependent transcriptional activation was detected between base pairs −2,373 and −2,316 relative to the translation start site. We also identified a required Sp1 binding site between −2,308 and −2,298. The data indicate that *Bmp2* is directly regulated by retinoic acid-bound receptors and Sp1.**

Bone morphogenetic proteins (BMPs)[1] are developmentally critical growth factors of the transforming growth factor-β family that were first described as having osteogenic activity in rats (1–3). *Bmp2* and *Bmp4* transcripts are widely expressed in vertebrate embryonic structures undergoing induction and morphogenesis (4–6). BMP signaling is involved in key embryonic processes such as epithelio-mesenchymal interactions (5), interdigital apoptosis in the developing limb (7), and dorsal-ventral axis specification (8). Mice having null mutations in the *Bmp2* or *Bmp4* (9, 10) or the Bmp receptor IA (11) genes die during early embryogenesis. The phenotypes of these mutants prove that BMP signaling is required for numerous extraembryonic and embryonic developmental processes.

The evolutionary conservation of the *Bmp2* and *Bmp4* genes and their *Drosophila* homolog *dpp* is remarkable. Conservation exists at both the functional and sequence levels (8, 12–14). Both BMP2 in mouse and DPP in *Drosophila* have pleiotropic functions and are expressed in a highly tissue- and stage-specific manner. Multiple promoters and alternative splicing produce three major and several minor *dpp* transcripts (15). Our work in murine cells and the *Drosophila* studies indicate that the multiple core promoters are closely involved in *Bmp2* and *dpp* tissue-specific regulation. It is likely that the regulation of this essential growth factor in mammals equals the complexity of *dpp* regulation in *Drosophila*.

Many *Bmp2*-expressing tissues develop abnormally in vitamin A-deficient embryos or after exposure to the potent teratogen retinoic acid (RA). These include the heart and cardiovasculature, limbs, central nervous system, craniofacial structures, and vertebrae (see Refs. 3 and 16 and references therein). The first indication that the *Bmp2* gene was regulated by RA was the discovery that it was strongly induced in F9 embryonal carcinoma cells stimulated to differentiate with RA (17). Subsequently, the *Bmp2* gene was found to be induced by RA in the developing chick limb (18). Since retinoid signaling may contribute to the normal pattern of embryonic *Bmp2* expression and since the aberrant induction of *Bmp2* by excess RA may cause some RA-associated deformities, elucidating the genetic regulatory elements controlling the RA inducibility of *Bmp2* will increase our understanding of normal development and teratogenesis.

F9 cells, a widely used model of cellular differentiation and early embryonic development, are an excellent biochemical system for investigating RA-inducible genes. F9 embryonal carcinoma cells differentiate rapidly and synchronously into primitive endoderm upon treatment with RA and into parietal endoderm upon treatment with RA and cAMP analogs (19). This model system has been used to identify retinoic acid response elements (RAREs) controlling the expression of several important developmental genes, such as *Hoxa1* (20), laminin B1 (21), and now *Bmp2*.

Here we describe the first genetic regulatory elements controlling the RA-regulated induction of the *Bmp2* gene in embryonic cells. RA-regulated gene expression is mediated by nuclear receptors, which act as retinoid-dependent transcrip-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF074942.

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[1] The abbreviations used are: BMP, bone morphogenetic protein; CAT, chloramphenicol acetyltransferase; CT, dibutyryl cyclic AMP and theophylline; RA, all-*trans*-retinoic acid; RACT, all-*trans*-retinoic acid, dibutyryl cyclic AMP, and theophylline; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RXR, retinoid X receptor; TK, thymidine kinase; bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; TTNPB, (*E*)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid.

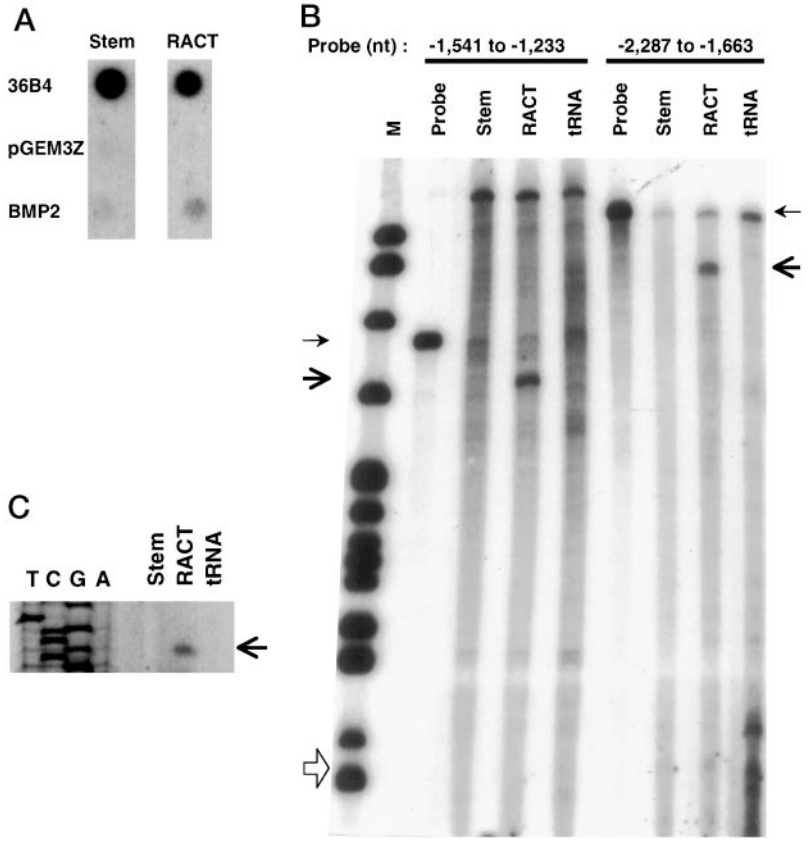

FIG. 1. **Identification of the RA-inducible *Bmp2* promoter.** F9 cells were grown for 72 (*A*) or 96 h (*B* and *C*) in the presence (*RACT*) or absence (*Stem*) of 0.5 (*A*) or 1 μM (*B* and *C*) RA and CT. *A*, nuclear run-on assays. ^{32}P-Labeled probes generated from transcripts initiated in these cells at the time of RNA extraction were hybridized to identical nitrocellulose strips spotted with a vector control (pGEM3Z) or plasmids containing the cDNA encoding a constitutive ribosomal protein (36B4) or BMP2 (pBMP2–452). *B*, ribonuclease protection assays. Antisense probes extending from nucleotide −1,541 to −1,233 and nucleotide −2,287 to −1,663 were hybridized to F9 cell RNA or to yeast tRNA, and ribonuclease protection assays were performed. The molecular weight marker (*M*) was pBR322 digested with *Msp*I. Visible fragments are 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, and 110 bp in length. The positions of the undigested probe and the protected fragment are indicated by the *thin* and *thick arrows*, respectively. The *open arrow* indicates the predicted size of a fragment generated by the proximal promoter used in osteoblast cells (36). *C*, primer extension assays. An antisense oligonucleotide corresponding to nucleotides −2,065 to −2,046 was hybridized to F9 cell RNA or to yeast tRNA, and primer extension was performed. One band was visible in the RACT lane only. A sequence generated from this oligonucleotide and pBMP2-NX is shown to the *left* of the primer extension lanes.

tion factors (22). Encoded by six different genes, RARα, -β, and -γ and RXRα, -β, and -γ, the receptors can act as homodimers and heterodimers, often with unique DNA binding and transactivation specificities (see Refs. 23 and 24). RARs bind to and are activated by all-*trans*-RA and 9-*cis*-RA, while the RXRs are activated only by 9-*cis*-RA. Our experiments in yeast suggest that, like *Hoxa1* and RARβ, ligands that bind both RARs and RXRs synergistically activate the *Bmp2* promoter. The work also suggests that, as in *Drosophila*, multiple transcription start sites are utilized in different mammalian tissues.

EXPERIMENTAL PROCEDURES

F9 Cell Culture and Differentiation—F9 embryonal carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum and 2 mM glutamine. The cells were induced to differentiate into primitive endoderm by adding RA alone and into parietal endoderm by adding RA, 250 μM dibutyryl cAMP, and 500 μM theophylline (RACT).

Library Screen—Using two sets of primers (5′-GAATTCCGGACTCAGGAGTG-3′ and 5′-CTCGAGACAGTCCAGCTGCG-3′ (GenBank™ accession number L25602)[2]; 5′-AAACAGTAGTTTCCAGCAGC-3′ and 5′-TCTGATTCACTAACCTGGTG-3′ polymerase chain reaction was performed on amplified aliquots of a genomic library in λ DASH II. Polymerase chain reaction-positive aliquots were then screened (25) with ^{32}P-labeled probe from the full-length *Bmp2* cDNA (pBMP2–452, kindly provided by David Israel) or a *Bmp2* subclone (pBMP2–68, nucleotides 6,483–6,724 with respect to the translation start site) to isolate two overlapping bacteriophage that contain the entire *Bmp2* gene and extensive 5′- and 3′-flanking sequences (16.5 kilobases total). λmBMP2–4771 contains the 5′-flanking region of *Bmp2* (nucleotides −8,583 to +3,360 relative to the translation start site). λmBMP2–1 contains the remainder of the transcribed region and the 3′-flanking region (nucleotides 2,979–15,700; see Fig. 2).

Plasmids—Plasmids were constructed as follows. All nucleotide positions are indicated with respect to the translation start site. For pCAT5′NN6.8 (−8,583 to −2,320) and pCAT5′NN6.3 (−2,287 to +3,360), *Not*I fragments extending from *Not*I sites in λ DASH II to each of two genomic *Not*I sites from λmBMP2–4771 were filled with Klenow fragment and ligated into the filled *Xba*I site in front of the pBLCAT2 Herpes simplex virus thymidine kinase (TK) minimal promoter (26). For pCAT5′XX4.5 (−3,367 to +1,206), a *Xba*I fragment was ligated into the *Xba*I site of pBLCAT2 in front of the pBLCAT2 TK promoter. For pCAT3′BB3.4 (9,318–12,700), a *Bam*HI fragment from bacteriophage λmBMP2–1 was filled and ligated into the *Sma*I site of pBLCAT2 downstream of the chloramphenicol acetyltransferase (CAT) coding region. For pCAT3′BN3.0 (12,700–15,700), a fragment downstream of the *Bmp2* gene was obtained by digesting bacteriophage λmBMP2–1 at the genomic *Bam*HI site and the λ DASH II *Not*I site. The ends were filled and ligated into the *Sma*I site of pBLCAT2 downstream of the CAT coding region. For pCAT4.5X (−3,367 to −1,658), a *Xba*I–*Xho*I fragment was ligated into the *Xba*I and *Xho*I sites of the promoterless vector, pBLCAT3 (26). For pCAT4.5XΔNot (−3,367 to −2,320; −2,287 to −1,658), two *Not*I sites at −2,320 and −2,288 in pCAT4.5X were digested to remove a 32-bp fragment and religated. For pCAT5′NB6.3B (−2,288 to −1,537), a *Not*I–*Bgl*II fragment was ligated into *Xba*I–*Bgl*II-digested pBLCAT3 after filling the *Not*I and *Xba*I ends. For pBMP2-H (−2,231 to −1,232), a *Bam*HI fragment was ligated into the *Bam*HI site of pBSIISK+ (Stratagene). For pBMP2-NX (−2,289 to −1,658), a *Not*I–*Xho*I fragment was ligated into the *Not*I and *Xho*I sites of pBSIISK+. For pΔss-BMP2 (−3,367 to −1,658), a *Sal*I–*Xho*I fragment from pCAT4.5X was inserted into the *Xho*I site preceding the *cyc1* promoter in the yeast reporter plasmid pΔss (27). For pΔss-SN1.05 (−3,367 to −2,316), a *Not*I–*Xho*I fragment in pΔss-BMP2 was removed and religated after filling the ends. For pΔss-BN.88 (−3,195 to −2,316), a *Bgl*II fragment from pGL-XN1.05 was inserted into the *Xho*I site of pΔss after partially filling the ends. For pGL-XN1.05 (−3,364 to −2,316), a *Xba*I–*Xho*I fragment from pCAT4.5X was inserted into the *Nhe*I and *Xho*I site of pGL2 (Promega) to make pGL-XX1.7, and then a *Not*I–*Xho*I fragment in pGL-XX1.7 was removed and religated after filling the ends. For pΔss-BB.83 (−3,195 to −2,369), a *Bgl*II–*Bam*HI fragment from pCAT4.5X was inserted into the *Xho*I site of pΔss after partially filling the ends. For pΔss-BB.06 (−2,373 to −2,316), a *Bam*HI–*Bgl*II fragment from pGL-XN1.05 was inserted into the *Xho*I site of pΔss after partially filling the ends. For pΔss-SB.18 (−3,367 to −3,191), a *Bgl*II–*Xho*I fragment in pΔss-BMP2 was removed and religated after filling the ends.

Nuclear Run-on Assays—F9 cells were untreated or treated 72 h with 0.5 μM RA, dibutyryl cAMP, and theophylline as described above. Nuclei were isolated and nuclear run-on assays were performed as de-

[2] D. Israel, personal communication,

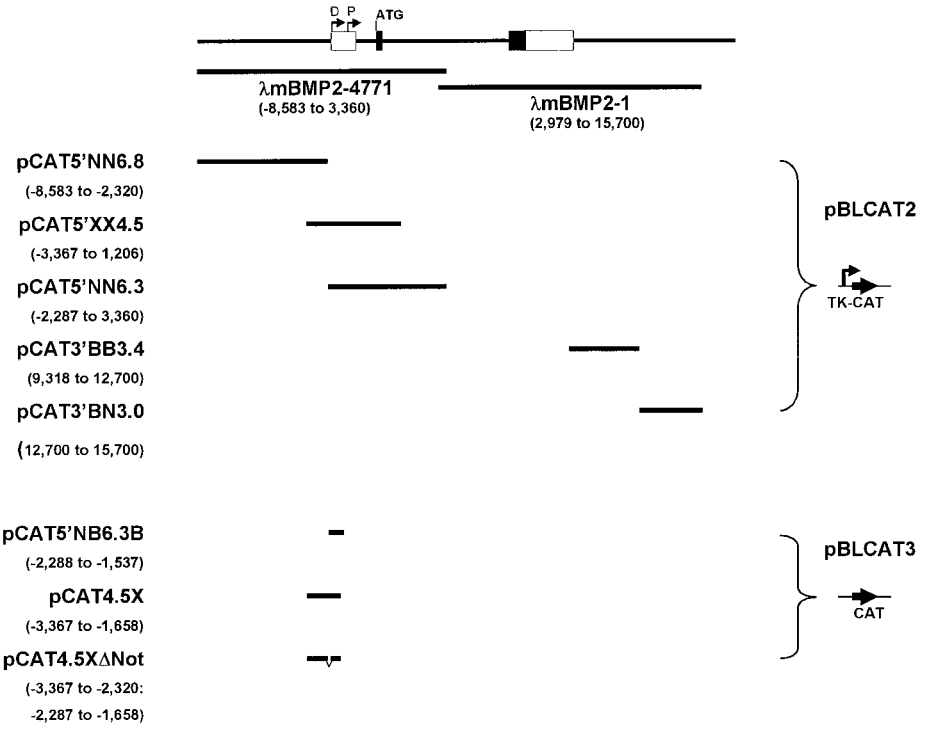

FIG. 2. ***Bmp2* gene structure and CAT reporter constructs.** A schematic representation of the *Bmp2* genomic sequence is shown. The sequence is numbered with respect to the translation start site (36). *Filled boxes* represent exons (36). *D* (distal) indicates transcription initiation from the RA-dependent promoter in F9 cells, whereas *P* (proximal) indicates the additional transcription initiation site utilized in osteoblasts (37). The *bars below* indicate the location and relative sizes of the sequences cloned into CAT reporter vectors pBLCAT2 and pBLCAT3. For pCAT5′NN6.8, pCAT5′-XX4.5, and pCAT5′NN6.3, fragments were inserted 5′ of the TK promoter in pBLCAT2. For pCAT3′BB3.4 and pCAT-3′BN3.0, fragments were inserted 3′ of the CAT coding region. For pCAT5′-NB6.3B, pCAT4.5X, and pCAT4.5XΔNot, fragments were inserted 5′ of the CAT coding region in the promoterless reporter vector pBLCAT3.

scribed previously (25, 28). Plasmids 36B4 (29), pGEM3Z (Promega), and pBMP2–452 were used for hybridization.

Sequencing—Sequencing was performed manually (25), by the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center (Tampa, FL) or by the DNA Sequencing Laboratory at the Interdisciplinary Center for Biotech Research (Gainesville, FL), using primers from the vector and internal sequences. Analysis of the RA-responsive upstream sequence for putative transcription factor binding sites was performed using TFSEARCH[3] *versus* the TFMATRIX transcription factor binding site profile data base (30) and by visual inspection. Promoterscan II[4] was used to located putative promoter sequences (31).

Primer Extension—Primer extension was performed as described previously (25). The primer 5′-GTGGGAAGCGCAGCGGCGGC-3′, corresponding to the complement of the sequence extending from −2,064 to −2,045, was labeled and hybridized to 29.7 μg of RNA and extended with avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.).

Ribonuclease Protection Assays—Ribonuclease protection assays were performed as described by Zinn *et al.* (32) with the following modifications. ^{32}P-Labeled RNA probes were made from pBMP2-H linearized with *Bgl*II and transcribed with T3 RNA polymerase and pBMP2-NX linearized with *Not*I and transcribed with T7 RNA polymerase. 10^6 cpm of each probe were hybridized to 10 μg of RNA overnight at 45 °C in 80% deionized formamide, 40 mM PIPES, pH 8.5, 400 mM NaCl, and 1 mM EDTA. After treatment with ribonuclease A, the product was electrophoresed on a sequencing gel.

F9 Cell Transfections and CAT Assays—All methods were essentially as described by Vasios *et al.* (21). F9 cells were cultured for 48 h without drugs or in the presence of CT, RA, or RACT, transfected by calcium phosphate precipitation, and then cultured an additional 24 or 48 h with drugs. All cells were cotransfected with the reporter gene and with pβAclacZ (21), which contains the β-galactosidase coding region driven by the constitutive β-actin promoter. Cell extracts were normalized for transfection efficiency as determined by β-galactosidase expression. Equivalent amounts of extract were incubated at 37 °C for 7 h with 250 mM Tris, pH 7.8, 5.3 mM acetyl coenzyme A, and 32.4 μM ^{14}C-chloramphenicol (51.5 μCi/μmol; NEN Life Science Products). After separation by thin layer chromatography (Whatman No. 4410221), chloramphenicol acetylation was quantified with a Molecular Dynamics PhosphorImager or a Beckman 60001C liquid scintillation counter.

Yeast Transformations and β-Galactosidase Assays—The pΔss β-galactosidase reporter vector (*URA3*) and the retinoid receptor expression vectors p2HG-RARβ (*HIS3*), pG1-RARγ (*TRP1*), and pG1-RXRγ (*TRP1*) have been described (27, 33). The reporter vector and various receptor expression vectors were used to transform the yeast strain BJ5409 (*his3, leu2, trp1, ura3*) using the lithium acetate method (34). Double or triple transformants were selected by plating on synthetic medium lacking the appropriate nutrients. For β-galactosidase assays, yeast cells were grown in selective medium in the presence and absence of retinoids for 24 h (all-*trans*-RA, Sigma; TTNPB and 9-*cis*-RA, Hoffman-La Roche; LG100268, Ligand Pharmaceuticals). The cells were lysed, and β-galactosidase activity was assayed by *o*-phenylphosphogalactopyranoside hydrolysis at 30 °C (25). Normalized β-galactosidase values were determined as follows: $(A_{420}/A_{600}) \times 1{,}000$/min of reaction time.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed essentially as described by Ausubel *et al.* (25). DNA probe was made by gel-purifying a 145-bp *Sau*3AI fragment (−2,372 to −2,227) from pCAT4.5X containing a Sp1 consensus sequence. The ends were filled in with Klenow fragment in the presence of [^{32}P]dCTP and [^{32}P]dGTP. Binding reactions contained 1 unit of rhSP1 (Promega), 60,000 cpm (2 ng) of probe, 10 mM HEPES, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM dithiothreitol, 0.25 mg/ml acetylated bovine serum albumin (New England BioLabs), 2% Ficoll, and 0.05 mg/ml sonicated salmon sperm DNA (Sigma). Samples were incubated for 30 min at room temperature and then loaded onto a 5% polyacrylamide gel (74:1 acrylamide:bisacrylamide, 5% glycerol (w/v)). Electrophoresis was performed at 300 V for 45 min in 0.5× TBE at 4 °C. The gel was dried under vacuum onto filter paper (Whatman) and exposed overnight to x-ray film (Eastman Kodak Co.).

RESULTS

Bmp2 Transcription in F9 Cells Increases in Response to RACT Treatment—RNA abundance may be regulated by alterations in transcription rate and in message stability. Previous experiments using the transcriptional inhibitors actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole showed that *Bmp2* mRNA stability does not change with RA treatment (35). This observation suggested, but did not prove, that RA increased *Bmp2* transcription rates. To test this hypothesis, we isolated nuclei from untreated cells or cells treated with RACT for 72 h and performed nuclear run-on assays (Fig. 1*A*). *Bmp2* gene transcription increased 3.7-fold in RACT-treated cells relative to untreated cells. In contrast, the transcription of 36B4, a constitutively expressed ribosomal protein, did not vary. This directly demonstrated transcriptional induction of the *Bmp2*

[3] TFSEARCH is available on the World Wide Web at http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html.

[4] Promoterscan II is available on the World Wide Web at http://biosci.cbs.umn.edu/software/promoterscan.htm.

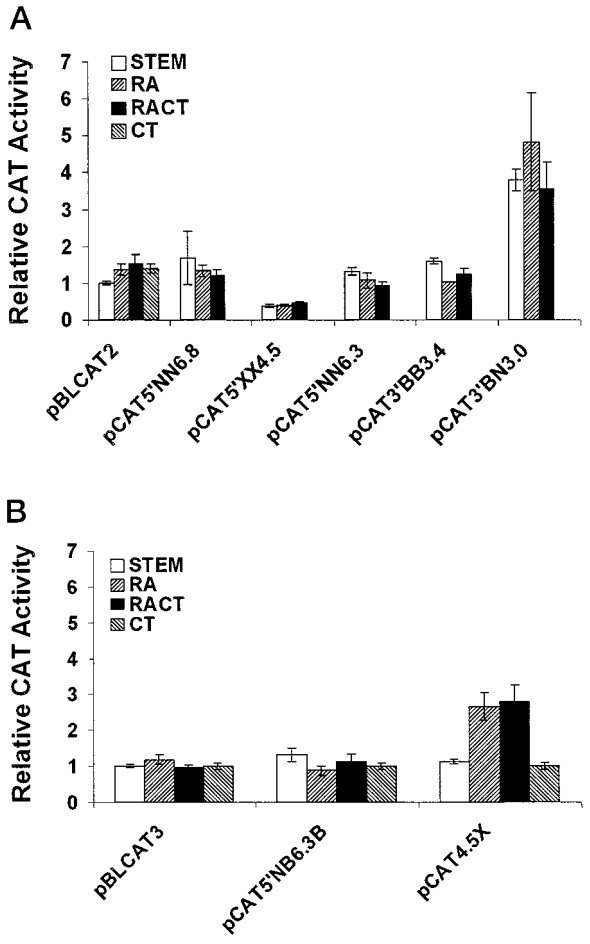

FIG. 3. **Regulation of *Bmp2* reporter activity in F9 cells.** F9 cells were grown for 48 h in the absence of drug (*Stem*) or the presence of 1 μM RA (*RA*) or 250 μM dibutyryl cAMP and 500 μM theophylline (*CT*) or all three drugs (*RACT*). Cells were then transfected with the plasmids indicated under each group of *bars* and pβAclacZ, which contains a constitutive promoter driving β-galactosidase. Reporter constructs containing the TK promoter are shown in *A*; constructs without the TK promoter are shown in *B*. 48 h later, cell extracts were made. β-Galactosidase assays were performed to normalize for transfection efficiency. Amounts of extract containing equivalent β-galactosidase activity were then used for CAT assays. Each *bar* shows the average of 3–6 experiments and the S.E. measurement.

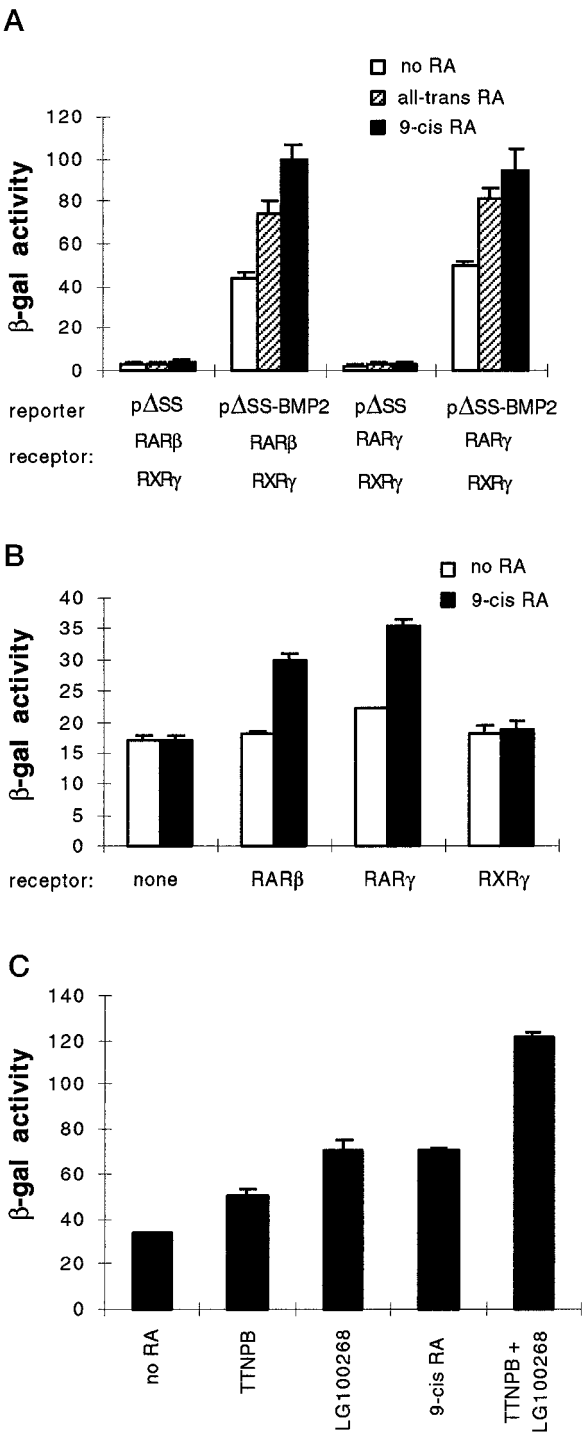

FIG. 4. **Regulation of *Bmp2* reporter activity in yeast.** Yeast strain BJ5409 was transformed with expression vectors encoding RARβ or RARγ alone or with RXRγ as indicated under each *bar* (*A* and *B*) or with RARβ and RXRγ (*C*). These yeast were subsequently transformed with the empty reporter plasmid pΔSS or pΔSS-BMP2 as indicated under each *bar* (*A*) or with pΔSS-BMP2 (*B* and *C*). Cultures were treated with the indicated retinoids at 1 μM. β-Galactosidase activities and S.E. measurements are shown. $n = 3$.

gene by RA.

Location of the Transcription Start Site—Two *Bmp2* promoters have been described in osteoblast cells (36, 37). We used ribonuclease protection assays to determine if these transcription start sites or others were RA-inducible in F9 cells (Fig. 1*B*). An antisense RNA probe that extended from nucleotide −1,541 to −1,233 relative to the translation initiation site was generated from pBMP2-H digested with *Bgl*II. The entire *Bmp2* probe was protected by RNA isolated from RACT-treated cells (Fig. 1*B*, *thick arrow*), indicating that *Bmp2* transcription initiated upstream of nucleotide −1,541 in F9 cells. No protected fragments were observed in the reactions containing RNA from untreated cells, yeast tRNA (Fig. 1*B*), or a sense probe extending from −2,230 to −1,233 (data not shown). The *open arrow* indicates the predicted location of a fragment generated by a transcript originating at the proximal promoter (nucleotide −1,344) described by Feng *et al.* (36). The absence of a fragment at this location indicates that, in contrast to osteoblast cells, this promoter is not used in F9 cells. Using an antisense probe that extended from −2,287 to −1,663 generated from pBMP2-NX, we observed a fragment of approximately 493 nucleotides. This suggests that transcription starts at approximately −2,156, near the distal promoter used in osteoblasts.

To confirm the transcription start site, primer extension was performed utilizing reverse transcriptase and a primer complementary to base pairs −2,064 to −2,045 relative to the translation start site (Fig. 1*C*). An extended product was detected only in the reaction containing RNA from cells treated with 1 μM RA and CT for 96 h and not in the reactions containing RNA

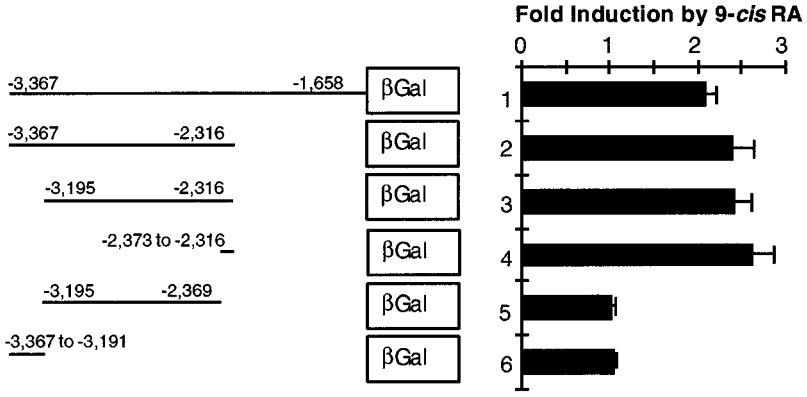

FIG. 5. **Deletion analysis of RA-responsive *Bmp2* sequences in yeast.** Yeast strain BJ5409 was transformed with expression vectors encoding RARβ and RARγ. These yeasts were subsequently transformed with various portions of *Bmp2* genomic DNA driving the β-galactosidase gene as indicated. Transcriptional induction by 1 μM 9-*cis*-RA is presented by -fold induction as indicated in the *histogram*. "1-Fold" induction indicates no difference in β-galactosidase activity between untreated and RA-treated cells. $n = 3$. *Bars* show S.E.

from untreated cells or yeast tRNA. Comparison with the genomic sequence designated a start site at nucleotide −2,127. No other RACT-dependent extended products were observed, suggesting utilization of one major transcriptional start site in F9 cells. The differences in mobility between RNA and DNA molecular weight markers explain the small discrepancy in start site position as determined by primer extension or RNase protection assays.

Regulation of Bmp2 Promoter Activity by RA in F9 Cells—CAT assays were used to detect RA- or RACT-dependent increases in CAT reporter activity driven by *Bmp2* genomic DNA in F9 cells. The reporter constructs containing sequence flanking the *Bmp2* gene are shown in Fig. 2. Since two transcriptional start sites have been described (37), all nucleotide positions are indicated relative to the translational start site (Fig. 2). Several large regions of the *Bmp2* upstream flanking region were inserted upstream of the Herpes simplex virus TK minimal promoter in pBLCAT2. These fragments, extending from −8,583 to −2,320 (pCAT5′NN6.8), −3,367 to +1,206 (pCATXX4.5), and −2,287 to +3,360 (pCAT5′NN6.3), failed to drive CAT expression in F9 cells treated for 96 h with 1 μM RA or 1 μM RA and CT (Fig. 3*A*). A fragment that included the 3′-end of the transcribed region (9,316–12,700; pCAT3′BB3.4) did not affect CAT activity. In contrast, a fragment distal to the 3′-end (12,700–15,700; pCAT3′BN3.0) caused a 3.5–4-fold CAT activation relative to the pBLCAT2 vector alone (Fig. 3*A*). Since activation occurred in cells treated with CT, RA, or RACT, this sequence must contain a non-RA-dependent regulatory element. Considering the highly tissue- and stage-specific expression of *Bmp2*, many regulatory elements are likely to control *Bmp2* expression.

Since developmental regulation of the *dpp* gene is mediated by the core promoter (38), we hypothesized that the RA responsiveness of the *Bmp2* gene was similarly controlled. If so, then the TK minimal promoter in pBLCAT2 might have interfered with RA-induced transcription. Therefore, *Bmp2* sequences were inserted into pBLCAT3, which lacks a minimal promoter. A 1,709-bp fragment, containing nucleotides −3,367 to −1,658 (pCAT4.5X), induced CAT activity 2.8-fold in cells treated for 96 h with 1 μM RA or 1 μM RA and CT relative to the activity observed in CT-treated cells (Fig. 3*B*). This fragment included 1,240 base pairs upstream of the transcription start site at −2,127. Finally, a fragment containing only 161 nucleotides upstream of the transcriptional start site (−2,288 to −1,537; pCAT5′NB6.3B) failed to induce CAT activity (Fig. 3*B*). These results are consistent with the presence of elements required for the RA response and promoter activity between nucleotides −3,367 and −2,288. As will be discussed below, we used a yeast reporter system to further delineate this RARE.

A Bmp2 RARE Drives RA-dependent β-Galactosidase Expression in Yeast—It is difficult to distinguish genes regulated directly by retinoid-activated receptors from those indirectly activated by other transcription factors induced by RA in mammalian cells. To avoid the complications associated with endogenous receptors and other transcription factors in F9 cells, we co-transformed yeast with mammalian receptor expression vectors and reporter genes driven by *Bmp2* genomic sequences. Although yeast do not normally express retinoid receptors, yeast transformed with receptor genes synthesize functional receptors. These can stimulate the RA-dependent expression of reporter genes controlled by mammalian RAREs (33, 39, 40). We inserted a fragment containing base pairs −3,367 to −1,658 of the *Bmp2* gene in front of the *cyc1* promoter and the β-galactosidase coding region of the yeast vector, pΔSS (27). The yeast strain BJ5409 was transformed with this plasmid and various combinations of RARβ, RARγ or RXRγ yeast expression vectors. Treatment of yeast expressing RARβ or RARγ and RXRγ with 1 μM all-*trans*-RA or 9-*cis*-RA induced β-galactosidase activity 1.7- and 2.3-fold, respectively, relative to untreated yeast (Fig. 4*A*). Yeast transfected with RARβ or RARγ alone and treated with 9-*cis*-RA also induced β-galactosidase activity 1.6-fold, indicating that the RAR homodimers could activate *Bmp2* nearly as efficiently as the RAR/RXR heterodimer (Fig. 4*B*). In contrast, yeast expressing RXR alone or yeast lacking receptors failed to express β-galactosidase in response to RA treatment (Fig. 4*B*). These experiments indicate that the RA responsiveness of this *Bmp2* sequence in yeast requires activation of RAR homodimers or RAR/RXR heterodimers.

In addition to the naturally occurring all-*trans*-RA, which activates only RARs, and 9-*cis*-RA, which activates both RARs and RXRs, several synthetic receptor-selective retinoids are available. TTNPB is often used to demonstrate RAR selectivity in mammalian cells because, unlike all-*trans*-RA, it cannot be converted to 9-*cis*-RA. LG100268 is an RXR-selective retinoid (41). We treated RARβ- and RXRγ-expressing yeast with 1 μM TTNPB, LG100268, or 9-*cis*-RA or the combination of TTNPB and LG100268. Like all-*trans*-RA, TTNPB activated transcription slightly (1.5-fold) but less effectively than 9-*cis*-RA (2.1-fold, Fig. 4*C*). Interestingly, the RXR agonist induced activity as effectively as the panagonist 9-*cis*-RA, which can activate both the RARs and the RXRs. Combined exposure to these retinoids stimulated activity by 3.6-fold (Fig. 4*C*). The synergistic activation of several RA-responsive genes by simultaneous ligand binding of each receptor subunit within a heterodimer has also been observed in mammalian cells (42, 43). These results are the first to demonstrate that the developmentally crucial *Bmp2* gene is activated directly by retinoid-bound receptors.

Having proven that this sequence could drive the yeast β-galactosidase reporter gene in a ligand- and receptor-dependent manner, we localized this element more precisely using a series of deletion constructs (Fig. 5). Deletions of 3′-flanking sequences up to position −2,316 and 5′-flanking sequences up to −2,373 do

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-3365 TCTAGAAACA GCCAGGGCCT AATTTCCCTC TGTGGGTTGC TAATCCGATT TAGGTGAACG
-3305 AACCTAGAGT TATTTTAGCT CCCCGACTGA AAAGCTAGCA CACGTGGGTA AAAAAATCAT
-3245 TAAAGCCCCT GCTTCTGGTC TTTCTCGGTC TTTGCTTTGC AAACTGGAAA GATCTGGTTC
-3185 ACAACGTAAC GTTATTCACT CTGGTCTTCT ACAGGAATGC TCAGCCCATA GTTTTGGGGG
-3125 TCCTGTGGGT AGCCAGTGGT GGTACTATGA AGGCTCCTGA ATGTAGGGAG AAATGGAAAG
-3065 ATTTCAAAAA AGAATCCTGG CTCAGCAGCT TTGGGGACAT TTCCAGCTGA GGAAGAAAAC
-3005 TGGCTTGGCC ACAGCCAGAG CCTTACTGCT GGAGACCCAG TGGAGAGAGA GGACCAGGCA
-2945 GAAAATTCAA AGGTCTCAAA CCGGAATTGT CTTGTTACCT GACTCTGGAG TAGGTGGGTG
-2885 TGGAAGGGAA GATAAATATC ACAAGTATCG AAGTGATCGC TTCTATAAAG AGAATTTCTA
-2825 TTAACTCTCA TTGTCCCCTC ACATGGACAC ACACACACAC ACACACACAC ACACACACAC
-2765 ATCACTAGAA GGGATGTCCA CTTTACAAGG TGTGTATCTA TGTTCAGAAA CCTGTACCCG
-2705 TATTTTTATA ATTTACATAA ATAAATACAT ATAAAATATA TGCATCTTTT TATTAGATTC
-2645 ATTTATTTGA ATATAAATGT ATGAATATTT ATAAAATGTA ATAATGCACT CAGATGTGTA
-2585 TCGGCTATTT CTCGACATTT TCTTCTCACC ATTCAAAACA GAAGCGTTTG CTCACATTTT
-2525 TGCCAAAATG TCTAATAACT TGTAAGTTCT GTTCTTCTTT TTAATGTGCT CTTACCTAAA
-2465 AACTTCAAAC TCAAGTTGAA TATTGGCCCA ATGAGGGAAC TCAGAGGCCA GTGGACTCTG
                                          RARE
-2405 GATTTGCCCT AGTCTCCCGC AGCTGTGGGC GCGGATCCAG GTCCCGGGGG TCGGCTTCAC
                                          Sp1
-2345 ACTCATCCGG GACGCGACCC CTTAGCGGCC GCGCGCTCGC CCCGCCCCGC TCCACCGCGG
-2285 CCGCCCCGTA GGGCGCGCCG TCCACACCCC TGCGCGCCGC TCCCGCCCGC CCGGGGATCC
-2225 CCGGCGCGCT GCGCCTCCGA GGGGGAGGTG TTCGGCCACG GCCGGGAGGG AGCCGGCAGG
                TATA                                    ↱
-2165 CGGCGTCTCC TTTAAAAGCC GCGAGCGCCG CGCCACGGCG CCTCCGTCGT CGCCGCCGGA
                                                PE  primer
-2105 GTCCTCGCCC TGCCGCGCAG AGCCCTGCTC GCACTGCGCC CGCCGCCGCT GCGCTTCCCA

-2045 CAGCCCGCCC GGGATTGGCA GCCCCGGACG TAGCCTCCCC AGGCGACACC AGGCACCGGA
-1985 CGCCCTCCCG GCGAAAGACG CGAGGGTCAC CCGCGGCTTC GAGGGACTGG CACGACACGG
-1925 GTTGGAACTC CAGACTGTGC GCGCCTGGCG CTGTGGCCTC GGCTGTCCGG GAGAAGCTAG
-1865 AGTCGCGGAC CGACGCTAAG AACCGGGAGT CCGGAGCACA GTCTTACCCT CAATGCGGGG
-1805 CCACTCTGAC CCAGGAGTGA GCGCCCAAGG CGAGCGGGCG GAAGAGTGAG TGGACCCCAG
-1745 GCTGCCACAA AAGACACTTG GCCCGAGGGC TCGGAGCGCG AGGTCACCCG GTTTGGCAAC
-1685 CCGAGACGCG CGGCTGGACT GTCTCGAG
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FIG. 6. **Nucleotide sequence of the upstream region of the *Bmp2* gene.** The sequence is numbered relative to the translational start site. The start site used in RA-treated F9 cells is indicated by an *arrow*. The sequence sufficient for RA-dependent activation in yeast is *underlined*. The Sp1 consensus sequence and a putative TATA box are in *boldface type*. The sequence complementary to the primer extension oligonucleotide is shown by a *heavy underline*.

not alter the induction by 9-*cis*-RA (*bars 1–4*). The reporter constructs containing only base pairs −3,195 to −2,369 or −3,367 to −3,191 were not induced by 9-*cis*-RA (*bars 5–6*). These results indicate that a 57-bp *Bmp2* promoter sequence located between −2,373 and −2,316 bp contains a RARE that is necessary and sufficient to induce RA-mediated transcription.

Sequencing and Analysis of the Upstream Region of the Bmp2 Gene—We sequenced base pairs −3,367 to −1,658 of the *Bmp2* gene to identify consensus sequences for other known regulatory proteins. Sequences consistent with a TATA-containing promoter sequence and a transcription start site at nucleotide −2,127 are depicted in Fig. 6. As shown in Fig. 1*C*, the primer extension assay confirmed the activity of this promoter in RACT-treated F9 cells. These features are consistent with a promoter at this site.

The sequence was also scanned for putative regulatory protein binding sites. A putative Sp1 site was identified between −2,308 and −2,298 (Fig. 6). Since others have demonstrated the importance of Sp1 sites for RA responsiveness (44–46), we deleted a 32-bp fragment containing the site (Fig. 2). This deletion failed to alter the magnitude of RA inducibility in either yeast (data not shown) or F9 cells (Fig. 7*A*). However, in F9 cells, both the basal and the induced transcription activity of the CAT reporter gene declined by 30% (Fig. 7*A*). We also demonstrated that recombinant Sp1 protein bound this sequence (Fig. 7*B*). These observations suggest that Sp1 influences the transcription activity of the *Bmp*2 gene but does not play a role in RA responsiveness.

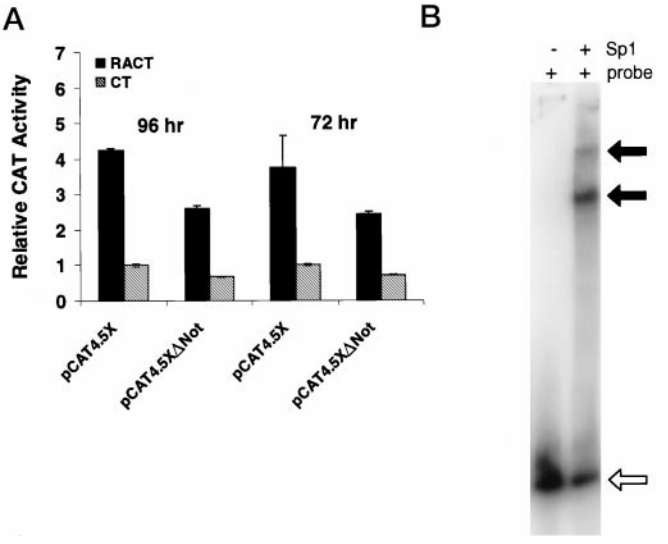

FIG. 7. **Sp1 protein binds a transcription activating sequence.** *A*, F9 cells were transfected with the reporter gene CAT driven by bp −3,367 to −1,658 (pCAT4.5X) or the same sequence lacking a 32-bp fragment containing an Sp1 consensus sequence (pCAT4.5XΔNot) as described in the legend to Fig. 2. Cells were extracted 48 or 24 h after transfection, resulting in 96 or 72 h of total exposure to drugs. The activity of these reporter genes was induced 4.2- and 3.9-fold by RA. However, both the induced and basal activity of the fragment lacking the Sp1 site was reduced approximately 30%. *Bars* show the range; $n = 2$. *B*, a 145-bp fragment containing the Sp1 site (−2,372 to −2,227) was end-labeled with ^{32}P-dCTP and ^{32}P-dGTP and bound to recombinant human Sp1. *Lane 1* indicates the migration of free probe (*open arrow*). *Lane 2* shows the retardation caused by binding of the DNA fragment to the unglycosylated (95-kDa) and glycosylated (105-kDa) forms of Sp1 (*closed arrows*).

DISCUSSION

Approximately 200 genes have been shown to be RA-responsive in one cell or another. Some genes are regulated directly by RA-bound receptors, *e.g. Hoxa1* (20), while others are secondarily regulated by other transcription factors modulated in RA-treated cells, *e.g. Fgf4* (47). Since both retinoid deficiencies and overdoses can cause embryonic malformations via the aberrant expression of key proteins controlling differentiation, proliferation, apoptosis, and morphogenesis, it is important to understand which genes are directly regulated. We now present evidence that the gene encoding the essential growth and differentiation factor, BMP2, is a direct target of RA.

Bmp2 is transcriptionally induced by RA in F9 embryonal carcinoma cells (Fig. 1*A*). Several pieces of evidence suggest that F9 cells utilize a *Bmp2* promoter initiating transcription at nucleotide −2,127 relative to the translation initiation site. First, ribonuclease protection assays indicate that the longest *Bmp2* transcript initiates near this site (Fig. 1*B*). Second, this

is the end of an RA-inducible primer extension product (Fig. 1*C*). Third, the predicted size of a transcript starting at −2,127 is consistent with the mRNA size of 3.8 kilobases (17, 36). Finally, sequences resembling a TATA-containing promoter are near nucleotide −2,127 (31).

In mouse osteoblasts, distal and proximal transcription start sites were observed at nucleotides −2,127 and −1,344 (36, 37). We did not observe a ribonuclease protection fragment corresponding to the proximal start site in untreated or in RACT-treated F9 cells (Fig. 2*B*). Thus, the proximal promoter does not mediate the RA-induced transcription of *Bmp2* in F9 cells. The existence of multiple *Bmp2* transcription start sites in different cell types is not surprising, because the expression of the *Bmp2* gene is highly dynamic. In addition, *dpp*, the gene encoding the *Drosophila* homolog of *Bmp2*, has three major and several minor transcripts produced from several promoters (15). Like the *dpp* transcript, the *Bmp2* transcript has an unusually long 5′-untranslated region of 1,125 nucleotides that might contain as yet uncharacterized regulatory elements. Since *Bmp2* and *dpp* are pivotal developmental genes, their spatial and temporal expression must be tightly regulated. We have demonstrated here that tissue-specific promoters are one mechanism involved in this tight regulation.

We have shown that this promoter and 1,709 base pairs of flanking region drive RA-dependent reporter gene expression in F9 cells. RA, which induces primitive endoderm differentiation, and RA and CT (dibutyryl cyclic AMP and theophylline), which induce parietal endoderm differentiation, caused equal activation of the CAT reporter gene (Fig. 3*B*). The endogenous *Bmp2* RNA is undetectable in undifferentiated cells and is induced modestly in RA-treated cells. In contrast, although CT does not induce differentiation and has no effect on *Bmp2* mRNA abundance, the combination of RA and CT induces the message abundance strikingly. Thus, sequence outside of −3,367 to −1,658 must contain the elements responsible for the synergistic activity of RA and cAMP.

Our demonstration that a 57-bp fragment of *Bmp2* genomic DNA can drive the expression of a β-galactosidase reporter gene in yeast transformed with retinoid receptors strongly suggests that this gene is directly regulated by receptor binding. The RAR/RXR heterodimer in the presence of ligands that activate both subunits activated the *Bmp2*-driven reporter most efficiently (Fig. 4*C*). Similar synergy has been observed for many genes in mammalian cells, including *Hoxa1* and the RARβ gene (42, 43). A requirement for specific receptor combinations and specific ligand activities may mediate the tight regulation of developmentally crucial genes such as *Bmp2*.

Known retinoic acid-responsive elements are highly polymorphic and conform loosely to the form of two repeated half-sites separated by nonconserved "spacer" DNA: RG(G/T)TCAN$_5$RG(G/T)TCA (22). Although the most frequent forms are direct repeats separated by 5 base pairs (N), some RAREs consist of inverted repeats, much wider spacing, and diverse arrangements of half-sites. Since the 57-bp *Bmp2* RARE lacks identity to any previously described RAREs, point mutational analyses will be necessary to identify the precise sequences bound by retinoid receptors. Considering that the numerous combinations of the six retinoid receptors and their various isozymes have distinct ligand- and DNA-binding specificities and that over 200 genes are known to be modulated in retinoid-treated cells (48), many more types of functional RAREs are likely to be found.

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Transcriptional Regulation of the *Bmp2* Gene: RETINOIC ACID INDUCTION IN F9 EMBRYONAL CARCINOMA CELLS ANDSACCHAROMYCES CEREVISIAE

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