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Evidence for two distinct retinoic acid response pathways for *HOXB1* gene regulation

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ABSTRACT **We have recently identified a promoter proximal retinoic acid (RA)-responsive site in the 5′ region of the *HOXB1* gene. In this report, we have identified the second RA-responsive site in the 3′ region of the *HOXB1* gene. This site also consists of a DR-2-type target of RA receptor·retinoid X receptor complex (DR-2B) and the binding site for a distinct RA-dependent coactivator termed retinoid-activating protein, which shows a different tissue-specific spectrum from the 5′ responsive site. This indicates that the activation of the *HOXB1* gene is achieved through two distinct pathways. These data define an unusual regulatory mechanism leading to the establishment of *HOXB1* gene expression.**

Vitamin A and its metabolic derivatives play critical roles in embryonic development, cell differentiation, and organ physiology. The *HOX* gene clusters can be regulated by retinoic acid (RA) treatment and thus may represent a major mediator of retinoid signaling. We have recently identified an RA-responsive site in the *HOXB1* promoter (7). This site is composed of two separable components, including a DR-2 motif, which is the direct target of a RA receptor·retinoid X receptor (RAR·RXR) heterodimer, and the upstream response element (URE), which serves as a binding site for an RA-dependent coactivator termed retinoid-inducible protein (RIP). RA induces synthesis of RIP in P19 cells but not in NT2/D1 cells. This difference in cell type-specific RA inducibility explains the failure of the *HOXB1* promoter to respond to RA in NT2/D1 cells. Although the transfected promoter fails to respond to RA, the endogenous *HOXB1* gene can still be activated in NT2/D1 cells (1, 2). The retention of inducibility of the intact gene strongly suggests the existence of a second pathway for RA responsiveness that is not mediated by the promoter.

In addition to *in vitro* inducibility by RA, the endogenous *HOXB1* gene shows two phases of expression in the developing embryo (3). In the primitive streak stage, the expression is restricted to the posterior half of the embryo, including the mesoderm and ectoderm. By early somite stage, *HOXB1* expression becomes divided into two domains: the posterior half of the embryo and the prospective rhombomere 4. Our preliminary analysis of transgenic mice harboring *HOXB1* reporter genes identifies the RA-responsive site in the promoter as critical in establishing the expression within rhombomere 4. However, the early and late phases of *HOXB1* expression have been reported to be sensitive to RA (4, 5). These lines of evidence strongly support the existence of another signaling pathway controlling the early expression of *HOXB1* gene in the posterior half of the embryo. The search for this pathway focused our attention on the 3′ portion of the gene and resulted in the identification of an RA-responsive element (RARE) composed of a DR-2-type motif whose activity is dependent on a flanking tissue-specific enhancer.

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MATERIALS AND METHODS

Plasmids. A DNA fragment containing the thymidine kinase (*TK*) luciferase gene was ligated into the *Sal* I site in pBluescript SK (+) vector (Stratagene) using the *Sal* I linker (pBS.TK.Luc). Various genomic DNA fragments derived from 3′ regions of *HOXB1* gene were inserted into the polylinker site of pBS.TK.Luc. TK.Luc.(DR-2B)$_1$, TK.Luc.(DR-2B)$_2$, and TK.Luc.(DR-2B)$_3$ were constructed by inserting DR-2B oligonucleotide (5′-CGGGCTGACCTTTTTACCTCGAAGCG-3′). A 90-bp DNA fragment amplified from the 3′ *HOXB1* genomic sequence by polymerase chain reaction (PCR) was ligated into these plasmids. HXB-Luc.(DR-2B)$_3$ was obtained by replacing the TK promoter region of TK.Luc.(DR2B)$_3$ with the *Spe* I–*Nco* I fragment derived from the *HOXB1* promoter region.

Cell Cultures and Transfection. Embryonal carcinoma cell lines P19 and NT2/D1 (6) and monkey kidney cell line CV-1 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Irvine Scientific). Transfection was performed by using calcium phosphate precipitation method as described (7). For the cotransfection assay, 0.1 μg of pCMX-hRARα and/or 0.1 μg of pCMX-hRXRα were used. The total amount of pCMX expression plasmids was kept constant by using parental pCMX plasmid.

RESULTS

Our analysis indicates that virtually the entire *HOX B* cluster, which is >100 kb long, is essentially free of repetitive DNA (unpublished). This unique sequence DNA continues 7 kb downstream of the 3′ end of the *HOXB1* gene, after which numerous repetitive sequence elements are found (Fig. 1*A*; data not shown). Constructs targeting the 7-kb region used in this analysis are shown in Fig. 1*A*. Note that the genomic DNA fragments derived from the 3′ region of this gene are ligated to a TK.Luc reporter in a fashion that preserves the natural downstream genomic configuration. When making these reporter constructs, we chose the promoter of the herpes simplex virus *TK* gene (*TK* promoter) to establish the independence of this regulation from the *HOX* promoter. As shown in Fig. 1*A*, construct 1 containing the *Sac* I fragment of +1.0 to +4.5 kb retained the same basal luciferase activity as parental Tk.Luc but was now activated 4-fold in response to 1 μM RA. The adjacent *Hin*dIII fragment of +4.0 to +5.5 kb (construct 2) did not confer responsiveness to RA, whereas construct 3 displayed weak responsiveness. Plasmid tkβRE.Luc, which has DR-5-type RARE from the RARβ2 promoter (8), was induced 11-fold. Parental plasmid TK.Luc was not affected by RA. We thus conclude that the +1.0- to +4.5-kb *Sac* I fragment may contain a RARE.

To localize this putative regulatory sequence, the *Sac* I fragment was subdivided into a series of nested constructs.

Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; RARE, RA-responsive element; URE, upstream response element; RIP, retinoid-inducible protein; TK, thymidine kinase; RAP, retinoid-activating protein.

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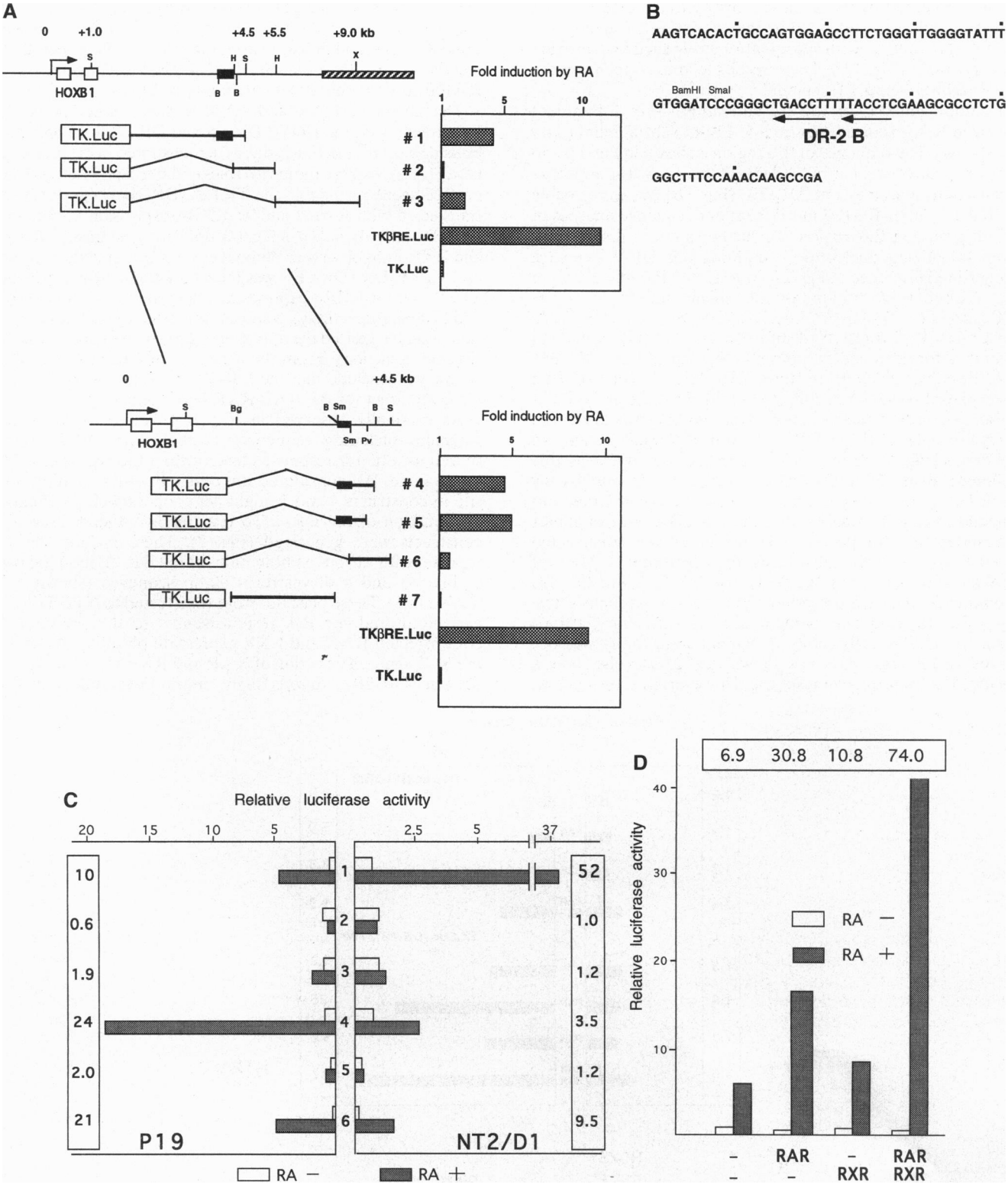

FIG. 1. RA-dependent transactivation by the *HOXB1* 3′ genomic region. (*A*) Localization of an RA-responsive site downstream of the *HOXB1* gene. Six different TK.Luc plasmids shown here were made by inserting various DNA fragments derived from the *HOXB1* 3′ region into the 3′ side of the TK.Luc gene and transfected into P19 cells. The hatched box in the map indicates the repetitive sequence and the filled box indicates the putative RARE. B, *Bam*HI; H, *Hin*dIII; S, *Sac* I; X, *Xba* I. Note that RA activates constructs 4 and 5, which share the 0.2-kb *Bam*HI–*Sma* I region shown as the thick line; constructs 6 and 7 remain unresponsive. (*B*) DR-2 motif in the 3′ RA-responsive site. Sequence analysis identified the DR-2 motif (DR-2B) indicated by arrows. An oligonucleotide was made from the underlined sequence for plasmid construction and the gel retardation assay. (*C*) DR-2B motif activates the *TK* and *HOXB1* (HXB) promoters from a downstream position. Activation profiles derived from six different plasmids are shown here. All plasmids were transfected to P19 and NT2/D1 cells. Numbers in boxes indicate fold induction by 1 μM RA. Plasmids: 1, TKβRE.Luc; 2, TK.Luc; 3, TK.Luc.(DR-2B)$_1$; 4, TK.Luc.(DR-2B)$_3$; 5, HXB.Luc; 6, HXB.Luc.(DR-2B)$_3$. (*D*) Synergistic action of RARα and RXRα on the DR-2 motif. TK.Luc.(DR2B)$_2$ was transfected into NT2/D1 cells with CMX-hRARα and/or CMX-hRXRα. Numbers in the box indicate fold induction by RA.

Results obtained by using these constructs indicate that the RA inducibility was encoded by the overlapping fragments in constructs 4 and 5, with marginal or no inducibility in fragments 6 and 7 (Fig. 1*A*). These results lead us to focus on the 0.2-kb *Bam*HI–*Sma* I fragment.

Deletion analysis of the 0.2-kb *Bam*HI–*Sma* I fragment localized RA responsiveness to a 5′ 110-nt subfragment (data not shown). The sequence of this region is shown in Fig. 1*B* and includes a direct repeat of 5′-AGGT(A/C)A-3′ (in antisense orientation) spaced by 2 nt (DR-2B) (Fig. 1*B*). We were unable to find any other RARE motif from our sequence analysis of 1.6 kb spanning this region. To further analyze RA responsiveness, an oligonucleotide containing this DR-2 sequence (underlined sequence in Fig. 1*B*) was ligated into the 3′ end of the TK.Luc transcription unit as single and triple copies [TK.Luc(DR-2B)$_1$ and TK.Luc(DR-2B)$_3$, respectively]. These constructs were transfected into P19 and NT2/D1 cells and examined for hormonal response. Following addition of 1 μM RA, the construct harboring three DR-2B motifs (Fig. 1*C*, lane 4) displayed robust inducibility (24-fold) in P19 cells, yet (for reasons described below) only a weak response was evident in NT2/D1 cells. A single DR-2 showed only weak activity in either cell (Fig. 1*C*, lane 3). The TKβRE.Luc control was able to induce 10- and 50-fold in P19 and NT2/D1 cells, respectively (lane 1), whereas the parental TK.Luc vector (lane 2) did not respond to RA. To test whether the DR-2B motif can confer RA inducibility to the *HOXB1* promoter, we constructed HXB(DR2)$_3$, which substitutes the promoter region of *HOXB1* (not including the URE or DR-2A) for the TK promoter. Though the promoter alone is only marginally active (lane 5), the response element confers efficient (21-fold) induction in P19 cells (lane 6). Similar inducibility was observed in F9 cells (data not shown); NT2/D1 cells show a positive but less effective response. However, as shown below, NT2/D1 cells contain low levels of RAR and RXR, and the response in these cells is markedly potentiated by cotransfection of RAR and RXR expression vectors. Together these results suggest that the *HOXB* DR-2B can function as an RARE in two embryonal carcinoma cell lines.

The ability of RAR and RXR to form either homo- or heterodimers on the *HOXB* DR-2A and DR-2B was confirmed by using a gel retardation assay, as demonstrated in the preceding paper (7). To explore the *in vivo* roles of the receptor heterodimer on *HOX* gene regulation, the TK.Luc.(DR-2B)$_2$ reporters were transfected with RARα and/or RXRα expression vectors into NT2/D1 cells. As shown in Fig. 1*D*, dramatic synergism of RARα and RXRα was observed. Without cotransfection of the receptor vectors, TK.Luc.(DR-2B)$_2$ was induced 7-fold by RA. Individually, RARα or RXRα expression enhanced the activity (31-fold and 11-fold, respectively), whereas cotransfection of both expression plasmids exerted the remarkable synergism on this construct (74-fold induction). From the activation and the DNA binding assays, we conclude that the DR-2B serves as a *bona fide* and effective target for the RAR·RXR heterodimer.

As originally observed in Fig. 1 *A* and *C*, a restriction fragment including sequences flanking the DR-2B motif served as a better response element than the synthetic DR-2 motif alone. We considered the possibility that an additional site in constructs 4 and 5 might act cooperatively to enhance the function of the DR-2. To test this hypothesis three new constructs were generated (Fig. 2). These include the *TK* reporter with various combinations of the DR-2B motif (shown as blocks) and a downstream 90-bp sequence (shown as a double line). These plasmids were transfected to NT2/D1 cells and monitored for RA responsiveness in the presence of cotransfected RAR and RXR expression plasmids. As shown in Fig. 2, synergistic action of RAR and RXR was observed on TK.Luc.(DR-2B)$_2$. Interestingly, more profound activation

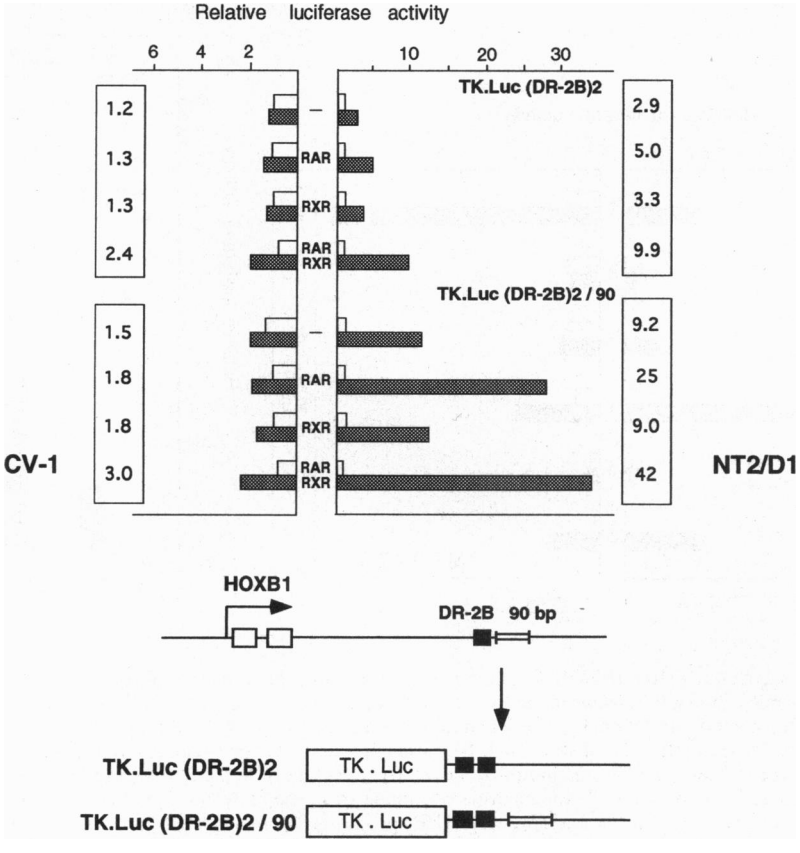

FIG. 2. Identification of a downstream RA coactivator. Two constructs [TK.Luc.(DR2B)$_2$ and TK.Luc.(DR2B)$_2$/90] were made as illustrated and transfected to NT2/D1 cells and monkey kidney CV-1 cells along with hRARα and/or hRXRα expression plasmids. Note that the 90-bp genomic fragment strongly augments the activation by RA in NT2/D1 cells but not CV-1 cells. Numbers in boxes indicate fold induction by RA.

was observed on TK.Luc.$(DR\text{-}2B)_2$/90. The luciferase activities from NT2/D1 cells transfected with the TK 90 construct (only the 90-bp fragment was inserted) were not affected by RA (data not shown). These results suggest an enhancing effect on DR-2 by the 90-bp fragment. This enhancing effect was also detected in P19 cells (data not shown).

These results suggest that the *HOXB* RARE activity may be augmented by an adjacent potentiating sequence. Interestingly, the 90-bp sequence failed to augment RA responsiveness in CV-1 cells with RAR and/or RXR expression vectors (Fig. 2). From gel retardation assays using the 90-bp region as probe, we have been able to identify a protein referred to as retinoid-activating protein (RAP) from NT2/D1 and P19 cell nuclear extracts (but not in CV-1 cell extracts) that specifically binds to this region (data not shown). This supports the transfection data and suggests that, in a fashion similar to the 5′ RARE, the unique combination of regulatory sequences may give rise to a cell type-specific RARE. Several reports have recently demonstrated that RAR and RXR have a high degree of cooperativity in binding target DNA and that heterodimer formation on a DR-5 motif strongly stimulates transcriptional activation (9–12). As shown in our preceding paper (7), the cooperative RAR–RXR binding was also observed on the DR-2A and DR-2B targets. Competition studies indicate these may be lower affinity than the heterodimer bound to a DR-5, which may explain the lower activation of a single DR-2 (2-fold) compared with RARβ2 RARE (10-fold) in P19 cells. However, three copies of either *HOXB* DR-2 produced a robust activation of the *TK* promoter. In NT2/D1 cells, cotransfection of RAR and RXR expression vectors dramatically increased RA inducibility through the DR-2. The *HOXB* DR-2B sequence closely resembles the RARE (5′-AGGTCAAAAGGTCA-3′) found in the promoter of mouse cellular retinol binding protein (13) and is distinct from the DR-5 response element identified in the 3′ region of *Hox-A1* gene (14). Our analysis indicates that though the DR-2s are effective binding sites they are ineffective response elements. Accordingly, they are ideally suited for cooperative interaction with other regulatory proteins.

DISCUSSION

Because many *HOX* genes are retinoid inducible, a major question to be resolved is whether this is a result of direct or indirect effects of these hormones. The work reported here identifies a DR-2-type RARE in the 3′ extragenic region of the *HOXB1* gene that serves as a direct binding site for the RXR·RAR heterodimer. This is distinct from the previously identified 5′ RARE in the promoter. Each of these enhancers is dependent on an adjacent coactivator that appears to confer the cell specificity and possibly the developmental specificity to the response elements. A model for this pathway of regulation is shown in Fig. 3. The DR-2A and DR-2B coactivators appear to be distinct proteins based on mobility shift assay, binding site preference, and expression patterns. The DR-2B potentiator (RAP) is constitutively expressed in NT2/D1 cells and P19 cells but not in CV-1 cells. In contrast, the URE binding protein RIP is induced by RA in P19 cells but not NT2/D1 cells. Thus, coactivation is achieved through two apparently distinct pathways that operate on similar principles. These cooperative interactions could be exploited in cell-specific and temporal regulation of the *HOX* locus, suggesting that 5′ and 3′ RAREs could function in different tissues and different time courses of the normal development process. Since the RARs and RXRs are essentially ubiquitous in their expression, the use of coactivators is a particularly effective way to restrict RA inducibility of the *HOX* locus to selective cell types and enables the different tissues to respond to RA in the different developmental time courses. Our transgenic mice data, supporting this hypothesis, also indicate that the 3′

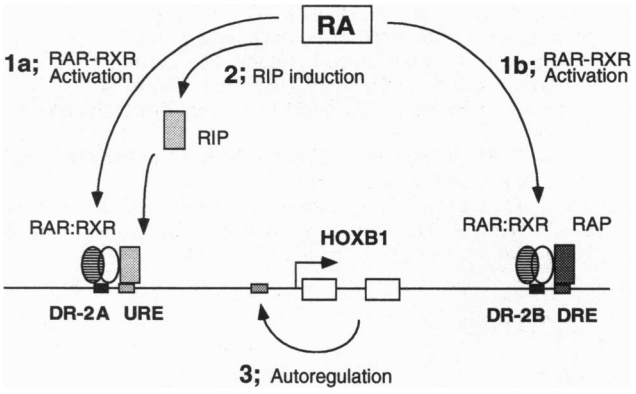

FIG. 3. Three RA signaling pathways. RA activates the *HOXB1* promoter through the 5′ or 3′ RARE. Both RAREs consist of two functionally separable components. The first components are the DR-2A and DR-2B sequences, which are direct targets for the RAR·RXR heterodimer (**1a** and **1b**, respectively). The second components are the cell-specific coactivators that modulate RA signaling. The 5′ coactivator RIP is strongly induced in P19 cells by RA and binds to a URE motif near DR-2A (**2**). RIP is not induced in NT2/D1 cells. The 3′ coactivator RAP, which binds to a DRE motif, is constitutively expressed in NT2/D1 and P19 cells but not in CV-1 cells. The third pathway is the autoregulation (**3**). Activation of the *HOXB1* gene forms the active autoregulatory loop that might maintain the expression triggered by RA.

RARE is required for the establishment of early *HOXB1* gene expression in the primitive streak stage embryo and that the 5′ RARE involves the establishment of rhombomere 4-specific expression (unpublished data).

It is important to note that *Hoxa-1* is also a paralogue of the *Drosophila labial* gene and is reported to have a DR-5-type RARE in its downstream 3′ region (14). The *Hoxa-1* gene is expressed in the early primitive streak stage, where the teratogenic doses of RA induce the expansion of its expression domain in a fashion similar to that observed for the *HOXB1* gene (3). These lines of evidence strongly suggest that the 3′ RARE is responsible for the establishment of the early phase of expression of *labial* paralogues and could be a common property shared by other *HOX* gene clusters. In contrast to the *Hoxa-1* gene, the *HOXB1* gene shows the unique and characteristic expression in the developing rhombomere 4 (3). Teratogenic doses of RA also expand the domain of this late-phase expression (3, 4), suggesting that RA could be involved in this process through the unique 5′ RARE in the promoter.

Together, these studies have identified two tissue-specific coactivators that enable the *HOXB1* gene to respond to RA in different cell types *in vitro* and *in vivo*. Isolation of coactivator genes will provide further understanding of the mechanisms controlling *HOX* gene expression in the developing embryo.

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