

The Direct Context of a Hox Retinoic Acid Response Element Is Crucial for its Activity*

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Tony Oosterveen, Patrick van Vliet, Jacqueline Deschamps, and Frits Meijlink‡

From the Hubrecht Laboratory, The Netherlands Institute of Developmental Biology, Uppsalalaan 8,
3584 CT Utrecht, The Netherlands

During embryogenesis, target genes of retinoid signaling are able to respond differently to identical concentrations of retinoids. Small differences in the retinoic acid response elements (RARE) may be essential for these distinct responses. Recently, we identified a RARE in a Hox enhancer (dubbed distal element) that is active relatively late during mouse development. We now show that the RARE motif in the distal element is necessary and sufficient for the induction of gene expression by retinoic acid (RA) in P19 embryonic carcinoma cells. Furthermore, the significance of these results was established by RA treatment of transgenic mouse lines carrying the distal element containing the wild-type or a mutated RARE. We compared the *in vitro* activity of the distal element-RARE with that of the direct repeat with 5-bp spacer RARE of the *RARβ2* gene, which is active during early during mouse development. We found that these RAREs, despite their similarity, responded differently to RA. By making single point mutations we show that the specificity resides in their retinoid X receptor-binding sites and is determined by base pairs located just outside the RARE consensus sequence. We suggest that the context of RARE motifs is important for the distinct transcriptional activities of genes under control of retinoid signaling.

During vertebrate development, retinoic acid (RA)¹ signaling is essential for the correct patterning of embryonic structures (1–3). There has been a long-standing debate whether or not RA acts in a gradient and provides in such a way positional information that is needed for the proper patterning of the embryo (4–6). Recently, evidence has been obtained that a graded distribution of RA in the hindbrain is essential for the specification of rhombomere identity (6). This process of rhombomere specification requires that distinct downstream gene targets of RA signaling must be able to respond differently to identical levels of retinoids.

RA-induced alterations in gene transcription are mediated

by nuclear receptors, the RARs (α , β , and γ) and RXRs (α , β , and γ), that act as ligand-responsive transcriptional regulators (reviewed in Ref. 7). RAR/RXR heterodimers bind to DNA through sequence motifs known as retinoic acid response elements (RAREs; Ref. 8). Like many other hormone response elements (HREs), the RAREs consist of two half-sites arranged in tandem repeats. The specificity of the HREs for a given nuclear receptor is determined by sequence, spacing, and relative orientation of these half-sites (7, 9). RAR/RXR heterodimers bind preferentially to direct repeats with the consensus sequence AGGTCA spaced by 1, 2, or 5 bp (DR1, -2, or -5; Refs. 7 and 10). Although naturally occurring RAREs have been identified that have divergent half-site configurations or sequence, the TCA motif is usually present (11, 12).

The binding of RAR/RXR heterodimers to the RAREs is co-operative. It is thought that the RARE motif aligns the RAR and RXR subunits and induces conformational changes that lead to stable interactions within this ternary complex (13, 14). Moreover, protein binding to one of the half-sites may induce structural distortions in the DNA that facilitate protein binding to the second site (14). These co-operative interactions suggest that the heterodimers do not pre-assemble before DNA-binding, but rather assemble on the appropriate RAREs. Beside the interaction in the ternary complex response elements also affect the interactions of the receptors with their ligands and other protein factors important for gene regulation (10, 15, 16). These possible allosteric modifications by the DNA-binding sites may cause a RARE-dependent transcriptional response upon ligand binding (16).

Transgenic experiments in mice reveal a difference in response of two distinct RAREs present in the Hox cluster. When in the *Hoxb4* early neural enhancer the DR5 RARE is replaced by a functional *Hoxb1* DR2 RARE, the expression pattern of the reporter construct in the neural tube acquires the features of those of *Hoxb1* (12). This experiment suggests that the RARE itself, and not its context, is crucial for the interpretation of the graded RA distribution.

Recently we discovered a novel functional DR5 RARE in a late neural enhancer, called the distal element (DE), which is able to mediate the expression of 5' *Hoxb* genes (17). This 533-bp DE drives reporter gene expression in a *RARβ2*-like pattern (17–19). Interestingly, the *RARβ2* promoter is under control of a DR5 RARE as well (20, 21). Although the late neural expression patterns are the same, the DE is not active in embryos younger than E10.0, whereas the *RARβ2* RARE starts to be active between E7.5 and E8.5 (17, 22). The distinct temporal activities of both RAREs could reside in sequence differences in the RARE themselves as both half-sites of the RAREs differ in one base pair from each other.

In this study we directly compared the activities of the *RARβ2* and DE-RAREs. We demonstrate in P19 embryonic carcinoma (EC) cells and mouse embryos a RA sensitivity of the

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‡ To whom correspondence should be addressed: Hubrecht Laboratory, Netherlands Inst. for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. Tel.: 31-30-212-1800; Fax: 31-30-251-6464; E-mail: frits@niob.knaw.nl.

¹ The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic response element; RXR, retinoid X receptor (9-cis retinoic acid receptor); DE, distal element; DR5, direct repeat with 5-bp spacer; EMSA, electrophoretic mobility shift assay; HRE, hormone response element; EC, embryonic carcinoma.

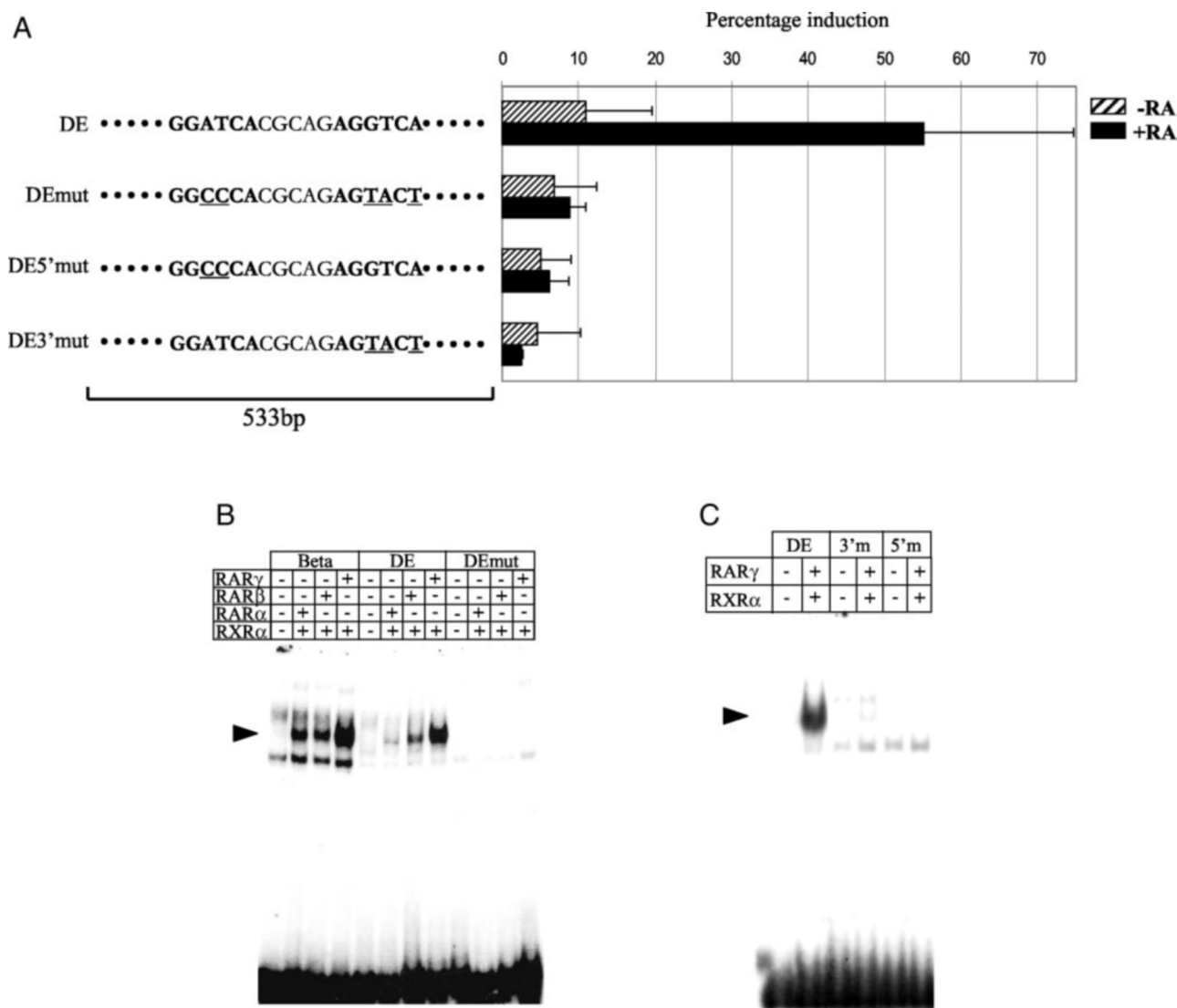


FIG. 1. RA-induced activity of the DE depends on the RARE. *A*, reporter assay of luciferase reporter constructs containing the wild-type or mutated versions of the 533-bp DE in P19 EC cells in the absence (–RA) or presence (+RA) of 1.0 μ M all-*trans* RA. The mutations shown (DEmut, 5'mut, and 3'mut) abolish the activity of the reporter construct and the binding of RAR/RXR heterodimers. Mutations in the RARE motif are *underlined*. The activity is given as percentage induction (\pm S.D.), obtained after dividing the means of the tested constructs over the mean activity of the empty luciferase construct. *B*, EMSA with 32 P-labeled oligonucleotides containing the RAR β 2 RARE (Beta), DE-RARE (DE), and a mutated DE-RARE (DEmut), as indicated on top. These oligonucleotides were incubated with extracts of COS cells transfected with an empty pSG5 vector or a pSG5 vector expressing one of the RA receptors. The presence or absence of the RA receptors is indicated by + and –, respectively. The arrowhead points to a specific complex formed only on the RAR β 2 and wild-type DE-RARE oligonucleotides, but not if the RA receptors are absent or when point mutations are made in the RARE motif of the DE. *C*, EMSA with labeled oligonucleotides containing the wild-type or mutated DE-RARE. These oligonucleotides were incubated with COS cell extracts in the presence (+) or absence (–) of the given RA receptors. The arrowhead points to a specific complex that is formed in the presence of the wild-type DE-RARE oligonucleotides but not in the absence of any RA receptor or if point mutations are made in either one of the half-sites of the DE-RARE.

DE, which depends on the presence of an intact RARE. We found that in P19 cells the DE-RARE is far less sensitive to RA than the RARE of the RAR β 2 gene. By comparing 25 bp RARE-containing elements derived from the RAR β 2 promoter and the DE, we found that the RXR-binding site and predominantly the direct context of these motifs account for the observed differences.

EXPERIMENTAL PROCEDURES

RA Exposure of Embryos in Utero, Whole Mount X-Gal Staining, and in Situ Hybridization—RA administration to pregnant mothers by oral gavage was performed as described (23). The procedures for the whole mount X-Gal staining and *in situ* hybridization with a *Hoxb5* probe have been described in Ref. 17. All animal experiments were conducted under the approval of the animal care committee of the KNAW (Royal Dutch Academy of Arts and Sciences).

DNA Constructs—The basic luciferase reporter construct used was a

modification of the p19Luc plasmid (24), which is named 19E1bluc and contains a fusion between the TATA box of the E1b promoter and the luciferase gene. Subfragments of the complete DE (25) and their mutated forms were cloned as *EcoRV*-*HincII* fragments into the *HincII* site of p19E1bluc. The subfragments of the DE studied were in most cases inserted in both orientations, but we found no influence of the orientation of these fragments on the activity of the resulting constructs. Therefore we only show the data corresponding to one orientation of the DE and its mutated versions. The DE with mutations in both half-sites was described previously (17), and the mutations made separately in each half-site were made in a similar way and checked by sequencing. Two copies of the 25-bp elements containing the RARE (see “Results” section for exact sequence) were cloned as *EcoRI*-*SalI* fragments in the *SalI* site of the p19E1bluc vector. These constructs were subjected to sequence analysis for verification.

Cell Culture and Transient Transfections—Culturing of P19 EC cells and cell transfections and luciferase and β -galactosidase assays were cultured as described by Folkers *et al.* (26, 27), with minor modifica-

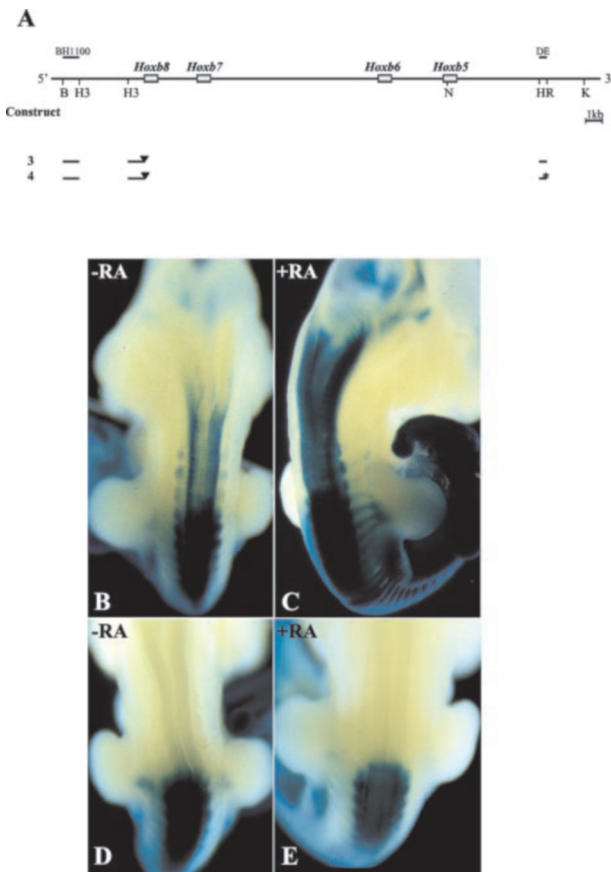


FIG. 2. Reporter gene induction *in vivo* by excess RA is mediated by the DE-RARE. A, schematic map of reporter constructs present in the genome of transgenic lines. A partial physical map of the *Hoxb* cluster with relevant *cis*-acting elements and restriction sites is shown on top. Construct numbers correspond to those used in Ref. 17. BH1100 is the enhancer that ensures posterior expression. Black arrowheads indicate the insertion site of the *lacZ* gene; the asterisk indicates the mutations in the two half-sites of the RARE. Restriction sites: B, *Bam*HI; H, *Hinc*II; H3, *Hind*III; K, *Kpn*I; N, *Nco*I; R, *Eco*RI. B–E, transgenic E10.5 embryos treated at E9.5 either with solvent (B and D) or with RA (C and E). B and C, transgenic embryos carrying *Hoxb8-LacZ* reporter construct 3 (see panel A) containing the DE and the BH1100 enhancer that is constitutively active in the posterior part of the embryo. D and E, transgenic embryos carrying *Hoxb8-LacZ* reporter construct 4 but with a mutated DE-RARE. Construct 3 and 4, constructs present in transgenic embryo.

tions. Cells were transfected in 24-well plates with 2 μ g of luciferase reporter construct and 1 μ g of pSV2lacZ. Lysates were prepared with 300 μ l of lysis buffer per well for 20 min at 4 °C, and 50 and 25 μ l of the lysate was immediately used for the luciferase and β -galactosidase assay, respectively. Results are the mean (\pm S.D.) of at least three independent experiments of three to four transfections for each construct. Relative induction was obtained by dividing the mean of each reporter construct by the mean of the empty luciferase construct. Percentage induction was calculated by dividing the activity of a particular construct by the sum of activities of all constructs in one experiment.

Electrophoretic Mobility Shift Assays (EMSA)—The EMSAs were performed as described (28). The sequences of the used oligonucleotides, labeled by filling in a 5' AGG sticky end, were as follows: β , AGGGTTCACCGAAAGTTCACGCA; DE, CGGGATCACGAGAGGTTCAGCAGAC; DEMut, CGGGCCACGAGTACTGCAGAC; DE3'm, CGGGATCACGAGTACTGCAGAC; DE5'm, CGGGCCACGAGTACTGCAGAC. The RARE sequences are shown in bold and the mutations are underlined. Whole cell extracts were obtained from COS-1 cells transfected with a pSG5 vector (29) expressing human *RARα*, *RARβ*, *RARγ*, or mouse *RXRα*.

RESULTS AND DISCUSSION

Upon RA treatment of P19 EC cells various downstream targets of retinoid signaling are activated, including *Hox* genes

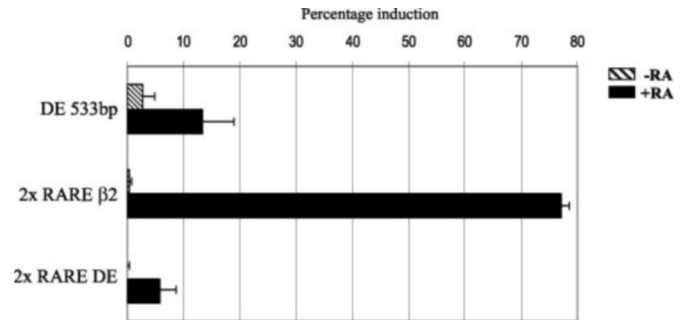


FIG. 3. Reporter assay comparing full-length DE to its isolated RARE and that of the *RARβ2* promoter, in presence and absence of RA. Constructs were transfected in P19 cells. Bars indicate luciferase activity in presence or absence of 1.0 μ M all-*trans* RA. The activity is given as percentage induction (\pm S.D.), obtained after dividing the means of the tested constructs by the activity of the empty luciferase construct mean.

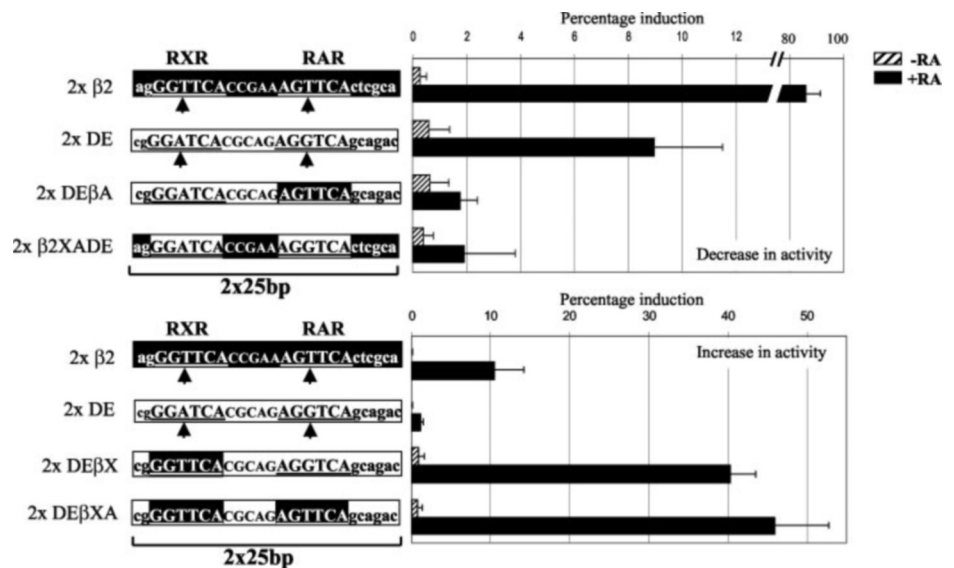
and the *RARβ2* gene. RA also induces differentiation of P19 EC cells in various differentiated derivatives including neural cells (30–33). Because the DE is particularly active in neural cells, we used this system to directly compare the activities of the RAREs derived from the RAREβ2 promoter and the DE.

RA Sensitivity of the Distal Element in P19 EC cells and Transgenic Mice Depends on the RARE—In P19 EC cells treated with RA, transcription of a luciferase reporter construct under control of the DE is weakly but significantly ($p = 0.05$) induced (Fig. 1A). To test whether the RA sensitivity was dependent on the RARE we made point mutations in either or both half-sites of the RARE. Mutations in both half-sites abolish *RAR/RXR* heterodimer binding (Fig. 1B, *DEmut*). EMSAs show that mutations in either of the half-sites (*DE5'mut* and *DE3'mut*) almost completely abolish *RARγ/RXRα* binding (Fig. 1C). As expected, the introduction of these different mutations in the DE resulted in loss of RA sensitivity (Fig. 1A). Taken together, these data demonstrate that the RARE motif is essential for the RA sensitivity of the DE in P19 EC cells.

To further confirm the *in vivo* relevance, we used transgenic mouse lines carrying a *Hoxb8-LacZ* reporter construct driven by the DE or *DEmut* (Fig. 2A; see Ref. 17). These constructs include an enhancer that drives the expression in more posterior embryonic regions (34) to allow us to verify that the integration site was permissive for expression. Previously we showed that *lacZ* expression in the hindbrain mediated by the DE depends on *Raldh2* (17). Now we investigated its sensitivity *in vivo* to an excess of exogenous RA. RA treatment at E9.5 of transgenic embryos carrying construct 3 (carrying an intact DE; see Fig. 2A) led at E10.5 to strong activation of the reporter gene in the hindbrain (Fig. 2, B and C). In contrast, similar treatment of embryos carrying construct 4, which contains the mutated DE (*DEmut*) did not result in *lacZ* induction (Fig. 2, D and E). Proper RA treatment was verified by analysis of *Hoxb5* response, and the results were confirmed in a second transgenic line carrying the same construct (not shown). These *in vivo* data confirm the significance of the RA response of the DE observed in the cell transfection assays.

The DE-RARE Is Sufficient to Induce RA-dependent Gene Transcription—We compared the RA-induced activities of the RAREs derived from the DE and *RARβ2* in P19 EC cells. We used an oligonucleotide of 25 bp containing the DR5 *RARβ2*-RARE and the flanking base pairs that are evolutionary conserved between mouse and human (18, 22). This particular element is sensitive to RA starting early during mouse development (22). The RA-dependent activity of two copies of this 25 bp element was much higher than that of an identical configuration of a 25-bp element including the DE-RARE and the

FIG. 4. Luciferase activity of constructs driven by different combinations of wild-type and mutant RAREs. The bars represent luciferase activities measured upon transfection of constructs in P19 EC cells in the absence (–RA) or presence (+RA) of 1.0 μ M all-trans RA. The constructs differ in the configuration of wild-type and mutant RARE half-sites and flanking sequences from the RAR β 2 (β 2') and DE control regions. The entire sequence of these 25-bp hybrid elements is given to the left of the figure. Sequences derived from the RAR β 2 RARE are shown in *reversed font* (white on black). The RARE motifs are *underlined*, and the arrows indicate the one-nucleotide difference in each half-site. The construct names are given on the left. The activity is given as a percentage induction (\pm S.D.), obtained after dividing the means of the tested constructs by the mean activity of the empty luciferase construct. The large range of values necessitated the use of two panels with different scales; note that in the upper panel the axis of the β 2 is interrupted.



same number of its flanking nucleotides (Fig. 3). The activity of the DE-RARE was significantly increased upon RA treatment ($p = 0.05$) but was lower than that of the complete DE both in the absence and presence of RA. The higher DE activity in the absence of RA is probably due to the presence of other (cryptic) binding sites for transactivators. These results show that other sequences in the DE may influence the activity of the RARE but that they are dispensable for its RA sensitivity.

The Distinct Activities of the RAREs of the RAR β 2 Gene and the DE Largely Depend on the Single-nucleotide Difference in the RXR-binding Site and on their Direct Context—In striking contrast with the large difference in RA sensitivity between the DE-RARE and the RAR β 2-RARE, the sequences of these DR5 RAREs differ by only one bp in each half-site. This should make it basically simple to pinpoint the nucleotides responsible for these distinct sensitivities. We therefore made a series of reporter constructs with two copies of the DE-RARE in which the corresponding RAR β 2-specific nucleotide change was introduced in either or both of the half-sites (Fig. 4). In addition, in one construct both of the half-sites of the RAR β 2-RARE were converted to a DE-RARE by two single point mutations.

As shown in Fig. 4, when the RAR-binding site of the DE was converted into that of the RAR β 2-RARE (construct DE β A) the RA sensitivity of this mutated DE was reduced to levels not significantly higher than that of the control (Fig. 4, –RA). In contrast, the transformation of the RXR-binding site of the DE into that of the RAR β 2 (construct DE β X) resulted in a very strongly enhanced RA sensitivity, to a level significantly ($p = 0.05$) higher than that of the RAR β 2-RARE. Surprisingly, converting both binding sites of the DE to the corresponding β 2-RARE sites (DE β XA) led to a level of sensitivity even marginally higher than that of DE β X, whereas conversion of the RAR site alone (DE β A) resulted in an element that retained only about 20% of the activity of the DE-RARE (DE). Accordingly, changing both half-sites of the RAR β 2-RARE into that of the DE (DE β XADE) led to a decrease in RA sensitivity.

We noted that without additional RA the DE β X and DE β XA elements had higher basal activities than DE and the RAR β 2 RARE. One possibility could be that the point mutations introduced in the RXR-binding site create a binding site for another transactivator. Because these motifs mediate high RA sensitivity, a more likely explanation is that they sense the RA present in the fetal calf serum (see Ref. 35). To test if the DE β X and DE β XA motifs can detect lower concentrations of RA than the

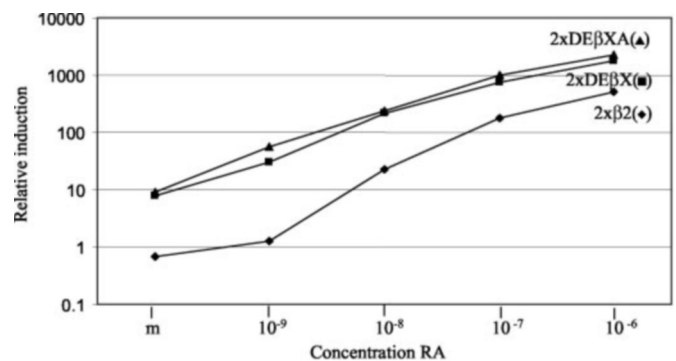


FIG. 5. Dependence on RA concentration of wild-type and hybrid RAREs. Reporter assays show relatively high sensitivity of hybrid elements DE β X and DE β XA to low concentrations of RA. Changing the DE RXR-binding site alone or together with the DE RAR-binding site into that of the RAR β 2 RARE increases the RA sensitivity in comparison to the RAR β 2 RARE in the native context. Luciferase activities are given as percentage induction, obtained by dividing the means of the tested constructs by the mean activity of the empty luciferase construct. These values are plotted (y-axis) against the different RA concentrations applied (x-axis). Note that both x- and y-axes are log scales.

RAR β 2-RARE we exposed cells transfected with these constructs to different concentrations of RA. DE β X and DE β XA respond to a RA concentration of 10^{-9} , whereas the RAR β 2-RARE respond only slightly to a RA concentration of 10^{-8} (Fig. 5).

Taken together, these data demonstrate that: (1) the single-nucleotide difference in the RXR-binding site of the DE and RAR β 2 RARE accounts to a large extent for the distinct activities; (2) the function of certain RAREs may be affected by their immediate flanking sequences; and (3) the activity of a response element not only depends on the sequence of the half-sites but also on their specific combination.

The Influence of the Immediate Context on the Activity of a RARE—The sensitivity of the RAR β 2-RARE starts between E7.5 and E8.5, whereas the DE starts to be active during later stages of mouse development (E10.0). RA concentration cannot be the major determinant in the timing of activation, because at E8.5 it is not the limiting factor. RA treatment of E8.5 transgenic embryos carrying a DE reporter construct does not activate the reporter at E9.5 (17). The RARE and its direct context might determine the stage-specific activity of this element. Consistent with this, the activity of the DE-RARE in P19

EC cells was only marginally lower when the rest of the DE was deleted.

How could the stage-specific activity of the DE-RARE depend on its direct context? Because there is no evidence that RAR/RXR heterodimers contact the flanking nucleotides of the RARE half-sites (13), this influence might be indirect. It is known that binding of RAR/RXR induces helical distortion in the DNA (14). Nucleotides outside the consensus sequence could affect the degree of helical distortions in the RARE and thereby influence its affinity and/or activity. Interestingly, it has been shown for several transactivators, including nuclear hormone receptors, that binding to DNA can induce allosteric modifications (16). Such modifications are known to influence the docking of co-factors of RA receptors thereby mediating specific transcriptional responses (10, 15, 36–38). Therefore, the degree of helical DNA distortions in the RARE motif, affected by nucleotides outside the consensus sequence, might result in allosteric modification of the RAR/RXR heterodimer. We propose that these modifications are necessary for the binding of particular proteins that are expressed during specific stages of development, which on their turn are essential for the activity of the RA receptors bound to the DE-RARE.

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