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A novel type of retinoic acid response element in the second intron of the mouse *H2K^b* gene is activated by the RAR/RXR heterodimer

Petr Jansa+ and Jirí Forejt*

Laboratory of Mammalian Molecular Genetics, Institute of Molecular Genetics, Academy of Sciences of Czech Republic, Vídenská 1083, 142 20 Prague 4—Krc, Czech Republic

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ABSTRACT

We have identified and characterized a novel retinoic acid (RA) response element (Hi-RARE) in the second intron of the mouse major histocompatibility *H2K^b* gene. The Hi-RARE sequence is conserved in all mouse classical and Q class I genes, in MHC class I genes of the rat, Rhesus macaque, cat and in the vast majority of human classical and non-classical class I genes. The Hi-RARE sequence lies within a regulatory element responsible for constitutive expression of a 5′ enhancerless *H2K^b* gene in the Ltk$^-$ fibroblasts. Hi-RARE consists of two inverted palindromic RARE consensus sites (5′-PuGGTCA-3′) separated by an 8 nt spacer. Mutational analysis revealed that both inverted palindromic hexanucleotide motifs are indispensable functional sites for the 9-*cis* RA response. The Hi-RARE sequence confers 9-*cis* RA inducibility to a heterologous promoter. The inducibility is further augmented in embryonal carcinoma cells by the expression of recombinant retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). *In vitro*, the recombinant RAR/RXR heterodimer creates DNA–protein complex with the Hi-RARE sequence. Treatment of P19 embryonal carcinoma cells with 9C-RA induces the Hi-RARE binding activity of nuclear proteins that proved to be RAR (or RAR-like)/RXR heterodimer. Thus the Hi-RARE represents a new type of RA response element with a role in the modulation of the expression of MHC class I family genes.

INTRODUCTION

The family of the mouse MHC class I genes is comprised of >25 genes, pseudogenes and gene fragments but only three of them represent the classical MHC class I genes both in mice (*H2K, -D, -L)* and in humans (*HLA-A, -B, -C)* (1). The variable number of related functional class I genes, *H2M*, *Q* and *Tla* in mice and *HLA-E, -F, -G*, and *-M* in humans, show a different pattern of expression (2–4).

The classical class I major histocompatibility complex (MHC) genes play a central role in the cellular immune response. Their products bind endogenously processed foreign polypeptides and present them on the cell surface. Thus cells carrying foreign antigen in the context of MHC molecules can be effectively discriminated from cells bearing self antigen during immunological surveillance by CD8$^+$ cytotoxic T lymphocytes (5,6). The expression of classical MHC class I genes varies greatly among cell types of an adult organism, being high in lymphoid tissues, liver and lung and low or absent on the surface of brain cells, acinar cells of the pancreas and mature sperm (7).

It is known that crucial control over the classical MHC class I gene expression is transcriptional (5,8). The region at nucleotides –213 and –61 relative to the transcription start site harbors three elements, enhancer A or class I regulatory element (CRE), enhancer B, and interferon response sequence (IRS) which partially overlaps the CRE. In the adult mouse, these elements control transcription of classical *H2* class I genes in both constitutive and inducible fashion. Enhancer A core region has been confirmed as a target for the binding of at least four transcription factors that may govern constitutive transcription of the *H2* class I genes: KBF1, H2TF1 and NFkB (reviewed in 9).

Inducible *H2* transcription by tumor necrosis factor and interferon was shown to be controlled from the CRE region and from the overlapping IRS in the 5′ flank of the *H2* class I genes (7). The upstream part of the CRE binds different members of the nuclear hormone receptor family (10) and acts as a response element for retinoic acid (11,12).

Although the majority of studies of MHC class I gene regulation have focused on the 5′ upstream regulatory sequences, it appears that these sequences do not account for all *cis*-regulation of these genes (13–15). In particular, we have shown that a deletion mutant of the *H2K^b* gene that lacks the entire 5′ enhancer sequence is fully expressed when transfected into L fibroblasts. We demonstrated further that its transcription was supported from a strong downstream regulatory element (H2DRE) located in the second intron and flanking exon sequences, +272 to +806 relative to the transcription start site (15).

Here we describe a new retinoic acid response element (referred to as Hi-RARE, histocompatibility-intron retinoic acid

* To whom correspondence should be addressed

+Present address: Division of Molecular Biology of the Cell II, German Cancer Research Center, 69120 Heidelberg, Germany

response element) within the second intron of the *H2K*^{b} gene DRE region. We show that retinoids induce binding of the nuclear proteins to Hi-RARE and that in embryonal carcinoma cells transcription of a reporter gene carrying the Hi-RARE sequence can be considerably augmented by simultaneous coexpression of the RAR–RXR transactivator heterodimers. Moreover, we demonstrate that Hi-RARE sequence binds recombinant RAR/RXR heterodimer *in vitro* and that monoclonal antibodies anti-RXR and anti-RAR nuclear receptors specifically supershift the complexes formed between Hi-RARE DNA and nuclear proteins of embryonal carcinoma cells. The Hi-RARE consensus sequence is conserved in mouse, rat, Rhesus macaque and human, thus supporting its functional role in various MHC class I genes.

MATERIALS AND METHODS

Plasmid constructions and oligonucleotides

The following double-stranded oligonucleotides, shown as coding strands, were used: Hi-RARE(W), wild-type form of the intronic (+559 to +584) sequence of the *H2K*^{b} gene, 5′-gatcGAGTGACCCCGGGTCGGAGGTCACGA-3′; Hi-RARE (M1), 5′-gatcGAGTGACCCCGGGTCG-AGGTCACGA-3′; Hi-RARE (M2), 5′-gatcGAGTGACCCCGccTCGGAGGTCACGA-3′; Hi-RARE (M3), 5′-gatcGGGTCGGAGGTCACGA-3′; Hi-RARE (M4), 5′-gatcGAGTGACCCCGGGTCG-3′; (Hi-RARE mutant forms 1–4). H-2RII, region II of the 5′ enhancer of *H2L*^{d} gene (–204 to –180), 5′-gatcAGGCGGTGAGGTCAGGGGTGGGGAA-3′. The double-stranded oligonucleotide RAREβ2 encompasses retinoic acid response element from the promoter of RARβ2 gene, 5′-TCGACGGGTAGGGTTCACCGAAAGTTCACTCGC-3′.

The *Dde*I–*Hin*fI (+517 to +684) and *Hin*fI–*Kpn*I (+684 to +806) fragments of the *H2K*^{b} gene DRE region were derived from p*Dde*I–*Kpn*I289conCAT plasmid (15) and were subcloned into the *Bam*HI site of the pUC18 cloning vector. The reporter plasmids pHiRAREconCAT, pHiRAREtconCAT were obtained by cloning a double-stranded oligonucleotide Hi-RARE as a monomer or tetramer, respectively, into the *Bam*HI restriction site of the pconCAT vector. Similarly have been prepared reporter plasmids carrying mutated Hi-RARE sequences. To construct the pDS245conCAT reporter, the *Dde*I–*Sau*3AI fragment (+517 to +762) overlapping the second intron of the *H2K*^{b} gene was subcloned into the *Bam*HI site of pconCAT (16). The pSS193conCAT reporter plasmid containing the *Sau*3AI fragment (–264 to –61) from the 5′ flank of the *H2K*^{b} gene was described previously (15).

Cell lines, transfections and nuclear extracts

Murine embryonal carcinoma P19 and F9 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (FCS). Murine fibroblast Ltk– cells and human cervical carcinoma HeLa cell line were cultured in DMEM containing 5% FCS. All media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cells were transfected 1 day after plating using the calcium phosphate coprecipitation technique with 5 μg of the chloramphenicol acetyltransferase (CAT) reporter plasmid and 2 μg β-galactosidase expression vector pCH110 (Pharmacia) as an internal control for transfection efficiency (16). The most efficient combination of transactivators was determined by cotransfection of the reporter plasmid with various combinations of pSG5 vectors expressing mRARα1, mRARβ2, mRARγ1 and/or mRXRα, mRXRβ, mRXRγ (a generous gift of P. Chambon). The cDNA of cloned receptors (17,18) was inserted into a cloning site of the pSG5 eukaryotic expression vector. The total amount of DNA used for transfection was adjusted to 10 μg with pUC18 DNA. After transfection the medium was replaced by a medium supplemented with all-*trans* retinoic acid or 9-*cis* retinoic acid (a kind gift of J. Grippo) at a final concentration of 10^{-8} to 10^{-5} M or with a vehicle (ethanol) and incubated for 20 h. CAT assays were performed as described previously (15) and normalized for transfection efficiency by measuring the activity of β-galactosidase. Nuclear extracts of the cell lines were prepared according Dignam and co-workers (19). Whole-cell extracts derived from cultured murine cell lines Ltk– and P19 were prepared according to the protocol of Schöler and co-workers (20). The extracts were used directly in electrophoretic mobility-shift assay or were stored at –80°C.

Electrophoretic mobility-shift assay (EMSA) and supershift experiments

The Hi-RARE double stranded oligonucleotide or DNA fragments were end-labeled by filling-in with [α-^{32}P]dATP using Klenow. Three to six μg of the cell extracts were incubated with 20–50 fmol radiolabelled probe ($0.5–2 \times 10^4$ c.p.m.) and with 1–2 μg of non-specific competitor poly(dI–dC) at room temperature for 20 min in a 20 μl reaction mixture containing 20 mM HEPES (pH 8.0), 70 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5% Ficoll (type 400). For competition an 100-fold excess of a specific oligonucleotide was added 5 min prior to the addition of the labeled probe. The EMSA experiments with the recombinant His-RARα/RXRα were carried out as described (21). Human 6H-RARα/RXRα (a gift of H. Stunnenberg) was expressed in HeLa cells from recombinant vaccinia virus. 6H-RARα/RXRα was copurified through Ni^{2+}-NTA chromatography (21). In supershift experiments a 1:40 dilution of monoclonal antibodies anti-RARα (Ab9α) and anti-RXR (mouse α,β,γ- 4RX-1D12), a gift from Pierre Chambon, was added to the binding reaction on ice, 15 min before loading on the gel. Samples were electrophoresed on 6% polyacrylamide gels in 0.25× TBE buffer at 25 mA at 4°C and DNA–protein complexes were then visualized by autoradiography.

Methylation interference assay

The *Dde*I–*Hin*fI fragment (+517 to +684) of the *H2K*^{b} gene subcloned in the *Bam*HI site of the pUC18 plasmid was radiolabeled at the 3′ end of either the coding or the non-coding strand and partially methylated with dimethyl sulfate (0.5%) at room temperature for 4 min as described (22). The probe (5 ng, 2×10^5 c.p.m.), twice ethanol precipitated, was incubated with 20 μg Ltk– nuclear extract proteins and with 5 μg poly(dI–dC) for 20 min and subjected to EMSA. Gel bands with bound and free probe were excised, eluted, extracted with chloroform, precipitated by ethanol and cleaved with 10% piperidine at 100°C for 30 min. After ethanol precipitation the probes were resolved on 8% polyacrylamide–8 M urea gel along with G/A reaction prepared according to the protocol of Maxam and Gilbert (22).

RESULTS

Ltk– nuclear proteins bind a new retinoic acid response element sequence (Hi-RARE) in the second intron of the *H2K^b^* gene

The 5′ enhancerless *H2K^b^* gene reveals a strong transcriptional activity dependent on an intragenic sequence called *H2* downstream regulatory element (H2DRE) (15). The *Dde*I–*Kpn*I fragment (+517 to +806) encompassing parts of the second and third exons and the whole second intron (+557 to +742) was shown to bear the main transcriptional activation potential of the H2DRE. To identify the DNA sequence that binds the Ltk– nuclear proteins we employed the methylation interference assay. Nine guanines on the coding strand and seven guanines on the non-coding strand interfered with the complex formation when methylated. The tightest contacts with DNA binding protein(s) were displayed by guanines 577 and 578 on the coding strand and guanines 580 and 582 on the non-coding strand (Fig. 1). Inspection of this DNA region for potential *cis*-acting regulatory sequences using the SIGNAL SCAN program (17) revealed the sequence motif AGGTCA (+576 to +581) which represents the half site of the consensus retinoic acid response element (RARE): PuG(G/T)TCA($N_{1–5}$)PuG(G/T)TCA (23). Furthermore, the sequence immediately upstream creates together with the above mentioned motif an everted repeat and a direct repeat, respectively, which we termed Hi-RARE (histocompatibility-intron RARE): 5′-TGACCC C GGGTCG G AGGTCA-3′ (+562 to +581).

Second intron of the *H2K^b^* confers retinoic acid responsiveness to the chloramphenicol acetyltransferase (CAT) reporter gene

To test the functionality of the Hi-RARE sequence, we constructed two reporter plasmids. The whole second *H2K^b^* intron with adjacent sequences (*Dde*I–*Sau*3AI fragment) was inserted in front of the CAT gene equipped with the chicken conalbumin promoter. The 9C-RA inducibility (10^{-8} and 10^{-6} M) of the resulting pDS245conCAT reporter construct was assayed after transfection into mouse P19 and F9 embryonal carcinoma cell lines and human cervical carcinoma HeLa cell line. In contrast to HeLa cells and F9 cells, P19 cells showed maximum CAT expression at the lower dose of 9C-RA (Fig. 2). The inducibility of the same construct in P19 cells was higher after 9C-RA treatment than after all-*trans* RA at the same concentration (10^{-8} or 10^{-7} M). This finding implies participation of the retinoid X receptors (RXR) in the transactivating event (24,25).

Hi-RARE-driven reporter gene is effectively transactivated by heterodimers of RAR/RXR nuclear receptors

To elucidate whether RA receptors are involved in transactivation, we cotransfected the pHi-RAREmconCAT reporter plasmid (with single copy of the Hi-RARE sequence) with various combinations of mouse RARs and RXRs expression vectors into P19 EC cells. The greatest response to 9C-RA induction was observed with the following combinations of RXR and RAR vectors: RARβ2-RXRγ > RARα1-RXRγ > RARα1-RXRα > RARβ2-RXRα (Fig. 3). While the expression of RAR homodimers did not elicit a significant response, the RXR homodimers induced small but reproducible increase of the reporter gene expression (Fig. 3, lanes 16–18). The pSS193conCAT reporter construct containing the 5′ *H2K^b^* gene enhancer (–254 to –61) with the previously described RARE (10) was co-expressed with the most efficient receptor combination (12) RARβ2/RXRβ (Fig. 3, lane 1) to serve as a positive control for the CAT activity. The control set of P19 transient transfectants with the same plasmid combinations as in Figure 3, but not treated with RA, did not exceed the values of the CAT activity found in negative controls (data not shown). The plasmid pconCAT did not show any response to 9C-RA when coexpressed with nuclear receptors RARα1 and RXRα (Fig. 3, lane 18). This finding implies that the Hi-RARE sequence inserted into the reporter plasmid (pHi-RAREconCAT) is responsible for the transactivation of the reporter CAT gene through binding of retinoic acid receptors.

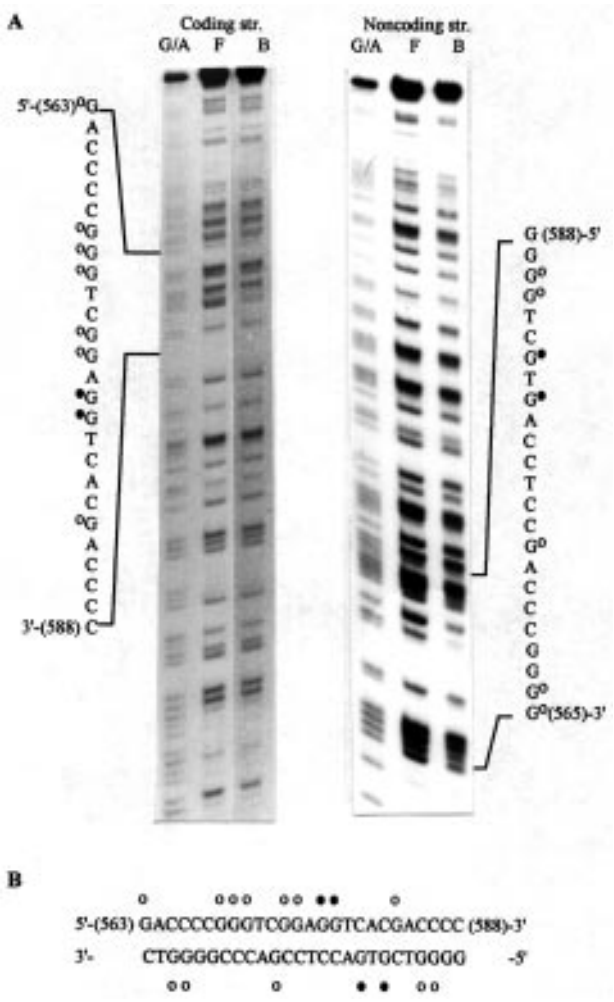

Figure 1. Methylation interference analysis of Ltk– nuclear proteins binding to the Hi-RARE of the *H2K^b^* gene second intron. (**A**) The coding or the non-coding strand of the *Dde*I–*Hin*fI fragment (+517 to +684 from the transcription start site of the *H2K^b^* gene) was partially methylated and used in binding reactions with the Ltk– nuclear extracts. The bound (B) or free (F) probe was recovered from the gel after electrophoretic mobility-shift assay procedure, subjected to chemical scission at positions of methylated guanines by piperidine and compared with the Maxam–Gilbert G/A sequencing reaction on 8% denaturing polyacrylamide gels. (**B**) The guanines whose methylation completely abolishes or partially interfere with protein binding are marked by black or open circles respectively.

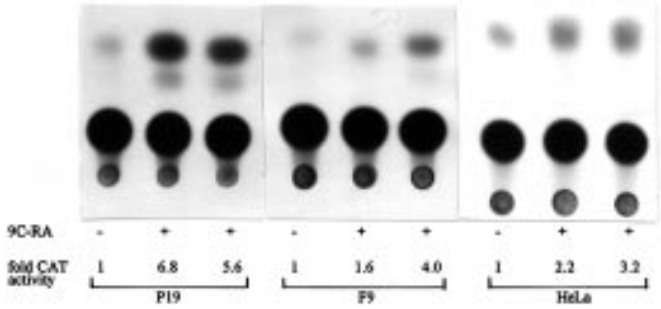

Figure 2. *H2K*^{b} intronic RARE mediates the RA responsiveness of a heterologous promoter in cell lines of different origin. Mouse P19 and F9 embryonal carcinoma cell lines and the human cervical carcinoma HeLa cell line were transfected with 5 µg of the pDS245conCAT reporter vector. The cells were treated with 9-*cis* retinoic acid for 20 h (10^{-8} and 10^{-6} M respectively) and the CAT assay was performed. The results shown are from a typical experiment and fold-induction values do not differ >25% between experiments.

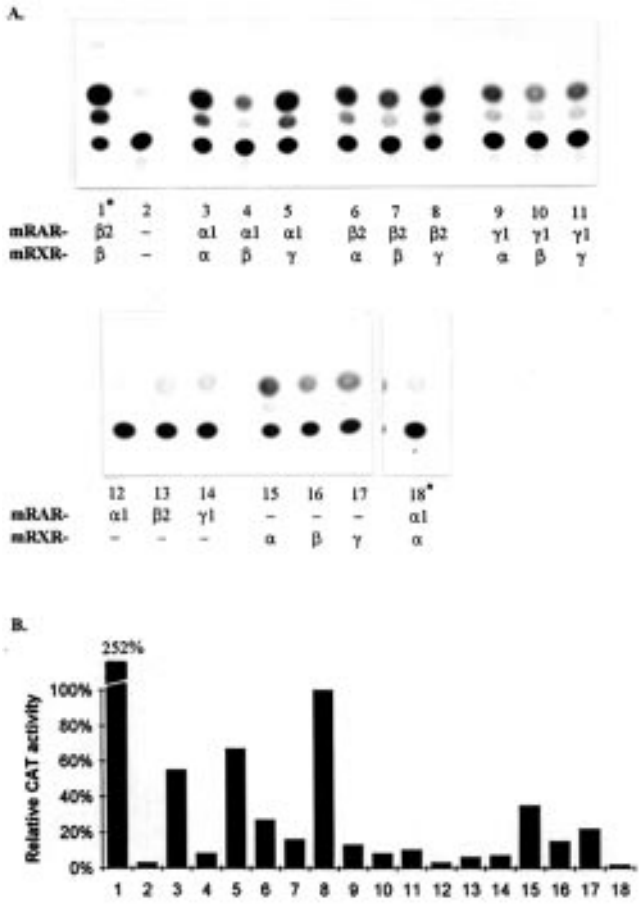

Figure 3. Differential transactivation of the pHi-RAREmconCAT reporter construct by co-expressed mouse retinoic acid receptors (α1, β2, γ1) and retinoid X receptors (α, β, γ) and RA treatment. (**A**) The reporter construct (5 µg) was cotransfected with 250 ng of the receptor-expressing vectors in various combinations into the P19 cell line as indicated. 9C-RA (10^{-6} M) was added, the cells were incubated for another 18 h, harvested and the CAT assay performed. The lane 1* shows the CAT activity of the pSS193conCAT in which the conalbumin promoter was under the control of the *H2K*^{b} gene 5′ enhancer. Co-expressed were expression vectors with mRARβ2 and mRXRβ. As a negative control, the parental reporter plasmid pconCAT was used as shown in the lane 18*. (**B**) The quantitative evaluation of the transactivation experiment shown in (A). The CAT activity of the pHi-RAREmconCAT with co-expressed RARβ2 and RXRγ receptors was taken as 100%. The results shown in the histogram correspond to average values of three independent experiments (±25%).

Both external half-sites of Hi-RARE are necessary for function

Mutations in the internal repeat or a deletion of the spacer guanine between the internal and 3′ repeat did not interfere with binding of 9C-RA-stimulated nuclear proteins, nor with the 9C-RA mediated response of CAT reporter vectors carrying these mutated Hi-RAREs (M1, M2, Fig. 4A). However, deletion of either 5′ or 3′ external repeat (M3 and M4 respectively, Fig. 4A) totally eliminated the nuclear protein binding to the mutated Hi-RAREs (data not shown) and their functional response to 9C-RA (Fig. 4B). The results strongly indicate that the Hi-RARE external half-sites are critical for its regulatory function. Evolutionary conservation of the external half-sites but not of the internal repeat (see below) seems to support such a conclusion.

Hi-RARE sequence specifically binds RAR/RXR heterodimers

Since the Hi-RARE sequence confers RA inducibility to a heterologous promoter and this inducibility is further augmented by expression of RX and RA nuclear receptors, we reasoned that binding of endogenous nuclear proteins to Hi-RARE might increase upon treatment with 9C-RA. To test this assumption, an electrophoretic mobility-shift assay was employed using the radiolabeled Hi-RARE oligonucleotide and nuclear extracts derived from treated and control P19 EC cells. Nuclear extract from untreated cells yielded three DNA–protein complexes in EMSA (Fig. 5A, lane 2). Following the 9C-RA treatment, the middle complex increased 5.7-fold in quantity and a new more rapidly moving faint complex occurred. In contrast, two major bands were not affected. Three out of four bands were entirely competed with 100 molar excess of cold Hi-RARE oligonucleotide over the radiolabeled Hi-RARE probe. The H-2RII oligonucleotide with the 5′ RARE of the *H2L*^{d} gene (10) displayed lower ability to compete. However, the RA-induced complexes were abolished by 100 molar excess of RAREβ2 oligonucleotide (Fig. 5A, lane 4), which represents natural RAR/RXR binding site of the RAREβ2 gene promoter (26). Since the mobilities of RA-induced complexes with Hi-RARE probe and with the radiolabeled RAREβ2 probe were identical (data not shown), it seemed likely that RA-induced complexes with Hi-RARE probe were created by RAR and/or RXR nuclear factors. The conclusion was confirmed by supershift experiments with antibodies against RXR and RAR. The RA-inducible complexes from P19 cells formed with Hi-RARE or RAREβ2 probes were specifically recognized by anti-RXR antibody as shown by the supershifts of specific complexes (Fig. 5B, lanes 4 and 9). Anti-RAR antibody decreased the amounts of RA-inducible complexes with both probes. The results confirm the presence of the RXR and the RAR or a related cross-reacting factor in the RA-inducible P19/Hi-RARE complexes.

To elucidate further the role of the Hi-RARE sequence as a target for binding of RAR/RXR heterodimer, we performed

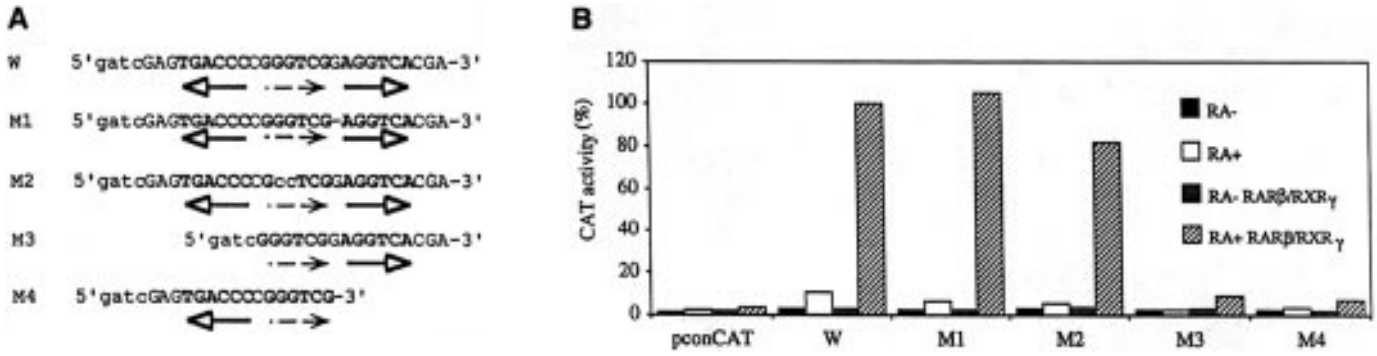

Figure 4. Mutational analysis of the Hi-RARE sequence reveals the functional importance of the outer RARE repeats. (**A**) The double-stranded oligonucleotides were used as competitors in EMSA and were ligated into pconCAT for transfection assays. W: wild-type of the Hi-RARE sequence of the*H2K*^{b} gene. M1, M2: mutations of the Hi-RARE affecting the inner (dashed arrow) RARE motif, either by deletion of the spacer G (M1) or by C–G substitution (M2). M3 and M4 are truncated Hi-RAREs with outer 5′ RARE motif deleted (M3) or 3′ RARE motif deleted (M4). (**B**) The effect of mutations in HiRARE sequence on the induction of CAT activity from the reporter plasmids. The pconCAT and the reporter constructs carrying wild-type (W) Hi-RARE and its mutated forms M1–M4 were transfected into P19 EC cells in the absence (RA–) or presence (RA+) of 9C-RA , without or with receptor-expressing vectors mRARβ2 and mRXRγ. CAT activity was scored as percent maximal conversion where the RA inducible activity from pHi-RAREmconCAT co-expressed with mRARβ2 and mRXRγ was set at 100%

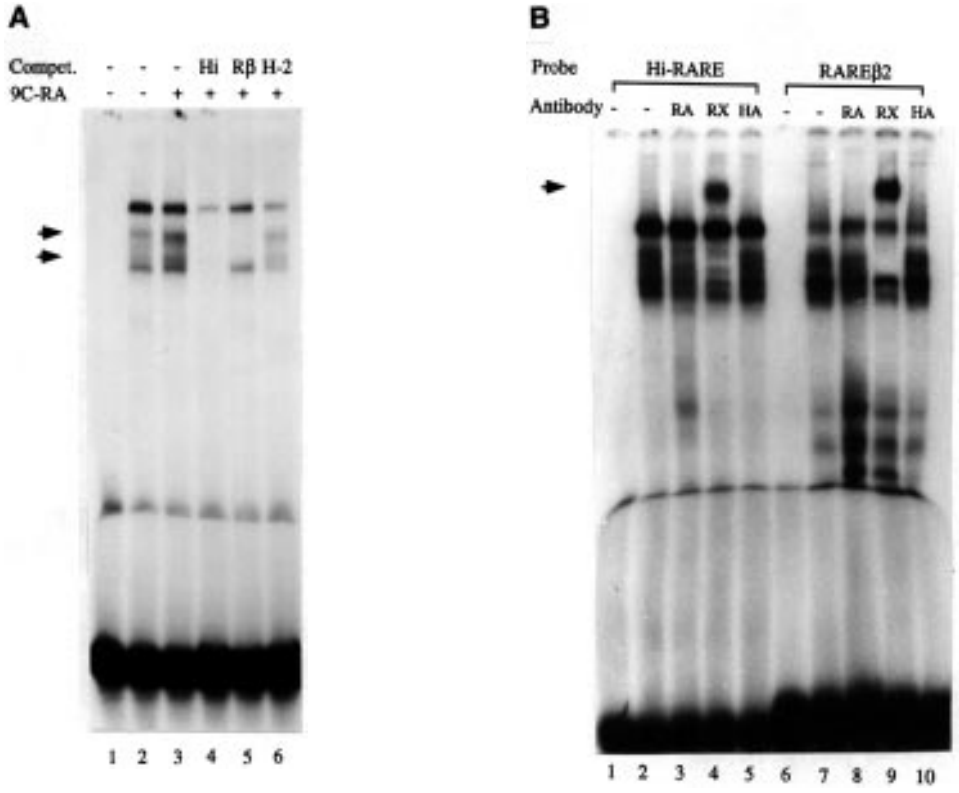

Figure 5. Identification of RA-inducible nuclear proteins in P19 EC specifically binding to Hi-RARE sequence. (**A**) Nuclear extracts (3 µg) prepared from P19 cells untreated (–, lane 2) or treated with 9C-RA (10^{-6} M) (+, lanes 3–6) for 24 h were used in binding reaction with ^{32}P-end-labeled Hi-RARE probe and subjected to EMSA. RA-inducible DNA–protein complexes (lane 3) are indicated by arrows. The specificity of DNA binding was tested by following oligonucleotide-competitors in 100 molar excess: Hi-RARE (Hi, lane 4); RAREβ2, a natural RARE from the promoter of RARβ2 gene (Rβ, lane 5); H-2RII, RARE derived from the 5′ enhancer of the *H2L*^{d} gene (H2, lane 6). (**B**) Supershift experiments with antibodies against RAR and RXR. The gel retardation assay was conducted using 3µg of nuclear proteins derived from P19 cells (treated with 9C-RA for 24 h) with the Hi-RARE probe (lanes 3–5) and with the RAREβ2 probe (lanes 7–10). Monoclonal antibodies against RAR (RA, lanes 3 and 8), against RXR (RX, lanes 4 and 9), and unrelated monoclonal antibody against hemagglutinin epitope (HA, lanes 5 and 10) were added to the mixture 15 min prior electrophoretical separation of the complexes in 6% polyacrylamide gel. The protein–DNA complexes specifically supershifted by anti-RXR antibody are indicated by arrow.

competitive EMSA using either the Hi-RARE probe or the RAREβ2 probe with copurified recombinant human His-tagged RARα/RXRα (6H-RARα/RXRα, a gift from H. Stunnenberg). The recombinant heterodimer displayed comparable binding affinity to Hi-RARE and to RAREβ2 sequences (Fig. 6, lanes 1–6). The critical parts of the Hi-RARE sequence for binding of

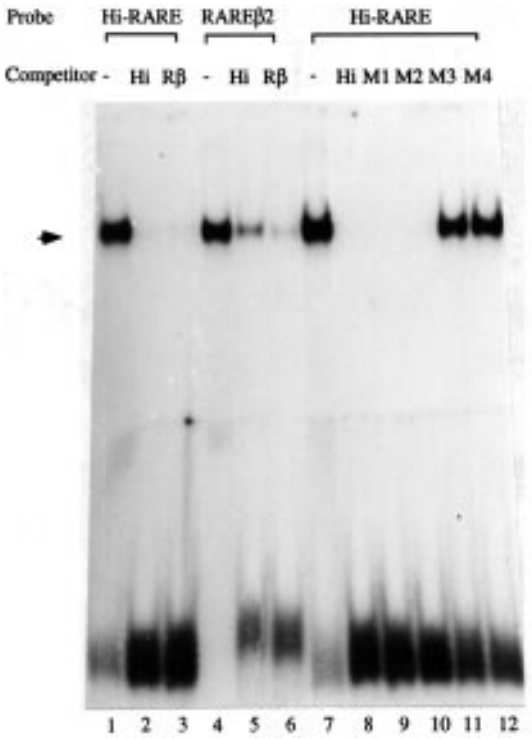

Figure 6. Recombinant RARa/RXRa heterodimer binds to Hi-RARE and to RAREβ2 sequences. EMSA was performed using ^{32}P-labeled Hi-RARE and RAREβ2 oligonucleotide probes with His-tagged RARα/RXRα heterodimer (15 ng). DNA binding specificities of the RAR/RXR heterodimer were determined by competitive binding reactions with the indicated oligonucleotides. Competitor oligonucleotides were added at 100 molar excess (Hi, Hi-RARE; Rβ, RAREβ2; M1–M4, mutated Hi-RARE see Fig. 4).

the RARα/RXRα were determined by competitive EMSA with a set of mutated Hi-RARE oligonucleotides. As shown in Figure 6 (lanes 7–12), the external hexanucleotide motifs of Hi-RARE spaced of by 8 nt are necessary and sufficient for binding the 6H-RARα/RXRα. Also the nuclear proteins derived from 9C-RA treated P19 cells required the external half-sites of Hi-RARE for successful binding (data not shown). Thus the requirement for external motifs for *in vitro* binding is consistent with the results of cotransfection experiments (see above).

Hi-RARE sequence is highly conserved in MHC class I genes of several mammalian species

If Hi-RARE plays a significant role in the regulation of the MHC class I genes then its sequence should be more conserved during evolution than adjacent, supposedly function-less intronic sequences. To test this possibility we aligned an 81 bp sequence from the second intron of the *H2K^b* gene that includes Hi-RARE plus 61 flanking nt, (+559 to +739) with the cognate intronic sequences of other mammalian MHC class I genes available from the GenBank database. The conservation of the Hi-RARE motifs, especially of the outer everted repeats (TGACCC and AGGTCA), was quite striking. None out of 16 functional classical mouse class I (*H2K*, *-D*, *-L*) and *H2Q* genes revealed a mutation which would violate the canonical PuG(G/T)TCA sequence of the outer repeats. A corollary to this finding is the relaxed conservation of the same sequences found in the mouse class I pseudogenes as five out of seven pseudogenes carried mutations that disrupted the consensus sequence of the Hi-RARE repeats. The Hi-RARE degenerate GGGTCG motif internal to the everted repeats is less preserved since it is disrupted in four mouse genes by single base substitutions. Thus the mouse Hi-RARE consensus sequence appears to be TGACCC(N8)AGGTCA.

The 3′ intron sequences flanking the Hi-RARE were more divergent than Hi-RARE and this tendency was even more obvious when human MHC class I genes were examined. The outer everted repeat was preserved in 14 out of 15 examined classical human class I genes and in seven out of nine non-classical class I genes and pseudogenes. The internal GGGTCG motif preserved in many mouse *H2* class I genes was altered in all their human homologues with the exception of *HLA-A1*. Moreover, all HLA class I genes examined contain an additional pentanucleotide insert, GCCNPu, inside the Hi-RARE so that the *HLA* Hi-RARE consensus sequence displays an 11–13 nt spacer: TGACCC(N11–13)AGGTCA.

The intact Hi-RARE was also conserved in MHC class I genes of the rat (27), rhesus macaque (28) and cat (29). In the rabbit (30) everted repeat was partially altered: TGACCC (N12) AGcTCg. In the dog (31), pig (32) and chicken (33) the Hi-RARE was mutated in both everted half sites but was still traceable. The choice of species examined was delimited by the availability of class I genes with the 2nd intron sequences in the GenBank database. A detailed sequence comparison of the 80 bp fragment carrying the *H2K^b* Hi-RARE with all studied MHC class I genes is available from the authors (jforejt@biomed.cas.cz).

DISCUSSION

In this report we identified and characterized a new putative retinoid response element, referred to as Hi-RARE, in the second intron of the mouse *H2K^b* gene. High steady-state levels of transcription had been observed previously from the 5′-enhancer-less *H2K^b* gene transfected into the Ltk– cell line. A novel enhancer-like activity responsible for this transcription was localized in the second intron and its flanking sequences (15). To analyze this activity further, we used nuclear extracts from the Ltk– cells in methylation interference assay and found protected guanines in the sequence: 5′-TGACCC C GGGTCG G AGGTCA-3′ at nucleotides +562 to +581. The sequence is composed of three core hormone response consensus half sites, PuGGTCA (17) indicating the presence of a response element to one or more members of the family of nuclear hormone receptors, including retinoid receptors RXRs and RARs, vitamin D3 receptor (VDR) and thyroid receptor (TH). These three consensus elements are arranged in a unique fashion as the outer motifs are in inverted palindromic arrangement and the third, degenerate site forms a spacer between them. The arrangement shows striking structural similarity to γF-RARE of the γF-crystallin gene (34).

The 5′-flanking regions of *H2* class I genes harbor the sequence functioning as RARβ/RXRβ response element (10–12,35) in embryonic cells and embryonal carcinoma cells. The intronic Hi-RARE also function as a retinoid response element as shown here by several independent experiments.

Retinoid hormones control gene expression by binding specific nuclear receptors which then function as ligand-activated transcription factors. These proteins bind to *cis*-acting RA response elements, RAREs, present in RA inducible genes. Two families of such receptors, RARs α, β, γ and RXRs α, β, γ and their

isoforms have been characterized in great detail (36,37). They bind as homodimers or heterodimers to RARE which consists of two or more directly repeated hexanucleotide motifs PuG(G/T)TCA. Binding of receptor homodimers follows, to a certain extent, the '5–4–3–1' rule (38–40) which requires a 5 nt spacer (DR5) between two directly repeated RARE motifs for binding the RARs, DR4 for thyroid receptors, DR3 for vitamin D3 receptor and DR1 for RXR homodimers. Thus, the internal degenerate motif together with the 3′ motif of the Hi-RARE may function as binding sites for the RXR homodimers. However, most of the receptors of the nuclear hormone superfamily form heterodimers, in many instances with RXR as an auxiliary protein (10,12,40,41). They generally require larger spacers and *in vitro* they bind well to synthetic RAREs including inverted repeats (42), thus potentially fitting in with the structure of the Hi-RAREs in the mouse and human MHC class I genes.

The capacity of Hi-RARE to mediate an RA response via retinoid receptors was suggested by the fact that the responsiveness to RA by the Hi-RARE driven reporter CAT gene was dramatically increased by cotransfection of RAR and RXR expression vectors. The most potent heterodimers were those involving RXRγ and RARβ2 or RARα1. RXR homodimers induced only a moderate response. This analysis strongly suggests that the Hi-RARE belongs to the family of retinoid response elements. This is further supported by the results of electrophoretic mobility shift and supershift assays which revealed that RA treatment of P19 cells induces nuclear proteins that bind to the Hi-RARE oligonucleotide and that these proteins are recognized by anti-RAR and -RXR antibodies. Moreover, the recombinant RAR/RXR heterodimer binds to the Hi-RARE with the affinity comparable with the RARE, its natural target in the promoter of the RARβ gene.

Taken together, the experimental data provide strong evidence that the Hi-RARE functions as a retinoid response element defined by its ability to confer RA responsiveness to a heterologous promoter. However, this evidence is not sufficient for determining the physiological function of the Hi-RARE at its autochthonous position in the *H2K*^{b} gene. The superfamily of nuclear receptors includes, besides RARs and RXRs, many other closely related proteins which can use each other's response elements rather promiscuously (17). Also, there is a number of orphan receptors, such as COUP, PPAR, MB67 (see 43 for review) for which the ligands are not known and which alone or with RXR as an auxiliary protein could contribute to the *H2K*^{b} gene regulation.

To attribute a functional role to a non-coding DNA sequence, its evolutionary conservation or diversification can be a critical clue. Based on this criterion, the fact that none out of 15 examined mouse class I genes carried any mutation in the 5′-TGACCC(N8)AGGTCA-3′ Hi-RARE motif uncovers the functional importance of this sequence element. In addition, five out of seven mouse class I pseudogenes show mutations that disrupted one or both inverted palindromic hexanucleotide motifs which is another piece of evidence that conservation of this sequence is necessary for a function of the *H2K*^{b} gene and not, for example, for some unrelated structural feature of a particular DNA domain. The computer assisted analysis revealed that a Hi-RARE with the same inverted palindromic core motifs is highly conserved also in human HLA genes, since the motifs are intact in 21 out of 24 HLA class I genes. The Hi-RARE sequence is also preserved in the second introns of MHC class I genes of

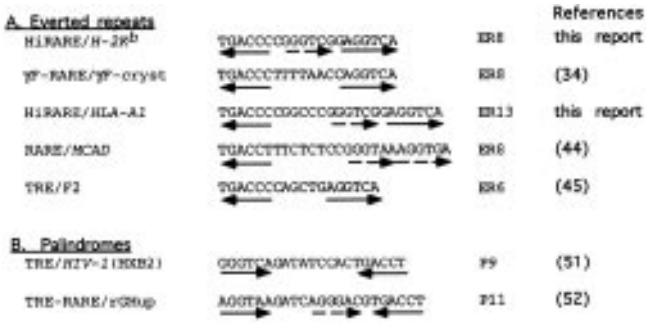

Figure 7. Sequence comparison of the *H2K*^{b} Hi-RARE with hormone response elements consisting of inverted palindromes or palindromes. (**A**) The inverted palindromes were observed in γ F-crystallin gene (γ F-cryst), human medium chain acyl-coenzyme A dehydrogenase gene (MCAD) and in chicken lysozyme silencer (TRE/F2). All these genes, as well as MHC class I genes, respond to RA induction. (**B**) Palindromic thyroid response elements were found in the long terminal repeat of the HIV virus, strain HXB2, and in the rat growth hormone promoter.

the rat, cat and rhesus macaque but not in the class I genes of rabbit, dog and pig. All non-rodent MHC class I genes analyzed in this study carried an insertion of the 5′-GCCNG-3′ pentanucleotide in their Hi-RAREs so that the consensus included the longer spacer: 5′-TGACCC(N11–13)AGGTCA-3′. The internal, partially degenerate motif 5′-GGGTCG-3′ is much less preserved in evolution. Thus the comparison of the second introns clearly points to the external repeats as being the most conserved parts of the Hi-RARE. This conclusion fits nicely with the mutational analysis of the Hi-RARE which showed that the deletion of either of the external inverted palindromic repeats results in loss of function of the mutated Hi-RARE.

Such a consensus, including two inverted repeats and a long internal spacer is far from the classical structure of RARE with two or more direct repeats and 1–5 bp long spacers (39,40). On the other hand, it closely resembles the structure of the γF-RARE of the γF-crystallin gene (34). Moreover, synthetic palindromic and inverted palindromic repeats are efficient transactivating response elements for RARs, T3Rs and VDRs and require longer spacers (3–12 nt) for optimal function (42).

Evidence is accumulating that genes other than MHC class I have inverted palindromic or palindromic RA response elements. Inverted palindromic repeats were described in the RARE of the promoter regions of the mouse γF-crystallin gene (34), the human medium chain acyl-coenzyme A dehydrogenase gene (44) and as a part of the chicken lysozyme silencer (45) (Fig. 7).

To conclude, it is conceivable that the Hi-RARE sequence in the second intron of the *H2K*^{b} gene represents a new type of RA response element. The role of retinoid receptors in this regulation may be further elucidated by determining steady-state levels of *H2* class I genes mRNA in mice with targeted loss-of-function mutations in RAR and RXR genes (46,47). The ultimate answer concerning the physiological function of this newly identified RARE element will require *in situ* mutagenesis of the *H2K*^{b} gene at the critical Hi-RARE sites.

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