Abstact. Synthetic peptides corresponding to cDNA-deduced amino acid sequences unique to the human and mouse retinoic acid receptor γ1 (hRAR-γ1 and mRAR-γ1, respectively) were used to generate anti-RAR-γ1 antibodies. Four mAbs were selected, which were directed against peptides found in region A1 (Ab1γ(Al)), region F (Ab2γ(mF) and Ab4γ(hF)) and region D2 (Ab5γ(D2)). These antibodies specifically immunoprecipitated and recognized by Western blotting RAR-γ1 proteins in COS-1 cells transfected with expression vectors containing the RAR-γ1 cDNAs. They all reacted with both human and mouse RAR-γ1 proteins, except Ab4γ(hF) that was specific for hRAR-γ1. Rabbit polyclonal antibodies, directed against a peptide from the mRAR-γ1 F region were also obtained (RPγ(mF)) and found to be specific for mouse RAR-γ1 protein. Furthermore, in gel retardation/shift assays the antibodies specifically retarded the migration of complexes obtained with a RA response element (RARE). Antibodies raised against regions D2 and F also recognized the RAR-γ2 isoform which differs from RAR-γ1 only in the A region. On the other hand, antibodies directed against the A1 region of RAR-γ1 (Ab1γ(Al)) only reacted with the RAR-γ1 protein. The antibodies characterized here allowed us to detect the presence of mRAR-γ1 and γ2 isoforms in mouse embryos and F9 embryonal carcinoma cells nuclear extracts. They were also used to demonstrate that the mRAR-γ1 protein can be phosphorylated and that the phosphorylation occurs mainly in the NH2-terminal A/B region.

Retinoic acid (RA),1 a vitamin A derivative, which is thought to be a natural morphogen (Maden, 1982;Thaller and Eichele, 1987), can act as a signaling molecule in a number of developmental systems. The pleiotropic effects of RA are likely to be mediated by specific nuclear receptor (RARs) which have been discovered in mouse and human (designated RAR-α, -β, and -γ) (Petkovich et al., 1987; Giguère et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Krust et al., 1989; Zelent et al., 1989). RARs belong to the steroid/thyroid hormone receptor superfamily, whose members act as ligand-inducible transcriptional enhancer factors (for reviews see Evans, 1988; Green and Chambon, 1988; Beato, 1989 and references herein) and can be divided into six distinct regions designated A through F (see Petkovich et al., 1987; Green and Chambon, 1988; Brand et al., 1988). The complexity of RARs has been further illustrated by the finding of multiple cDNA isoforms for each RAR (Krust et al., 1989; Giguère et al., 1990; Kastner et al., 1990; Leroy et al., 1991; Zelent et al., 1991). For each RAR gene (either α, β, or γ) the corresponding isoform messenger RNAs are generated from two promoters and differential splicing of exons encoding the A region.

Specific spatial and temporal patterns of distribution of the RAR-α, -β, and -γ transcripts have been demonstrated in adult mouse tissues (Krust et al., 1989; Zelent et al., 1989; Kastner et al., 1990) and during mouse embryogenesis (Dollé et al., 1989,1990; Ruberte et al., 1990, 1991). In particular, the localization of RAR-γ transcripts during embryogenesis as determined by in situ hybridization, suggests that RAR-γ plays an important role during early morphogenesis and differentiation of cartilage and cornified squamous epithelia (Dollé et al., 1989, 1990; Ruberte et al., 1990, 1991). Furthermore, the two predominant RAR-γ isoforms, RAR-γ1 and RAR-γ2, appear to be differentially expressed in adult tissues and during the course of embryogenesis, as determined by Northern blot analysis (Kastner et al., 1990).

In this paper, we describe the preparation and characterization of rabbit polyclonal and mouse mAbs directed against synthetic peptides specific to mouse and/or human RAR-γ isoforms. These antibodies specifically immunoprecipitate and recognize by Western blotting mouse or human RAR-γ in cells transfected with expression vectors containing the corresponding cDNAs. They also specifically retard the
migration of RAR-γ/RARE (RA response element) complexes in gel shift assays. Additionally, these antibodies allowed us to detect the presence of RAR-γ isoforms in F9 embryonal carcinoma cells and mouse embryos, despite the low amount of these proteins in such cells and tissues. Finally, using our antibodies, we have been able to demonstrate that the RAR-γ protein is posttranslationally modified by phosphorylation.

Materials and Methods

DNA Constructs

The plasmids containing the mouse or human RAR gene coding sequences RAR-α1, RAR-β2, and RAR-γ (previously referred to as RAR-α0, RAR-β0, and RAR-γ0, respectively), were as described (Petkovich et al., 1987; Brand et al., 1988; Krust et al., 1989; Zelent et al., 1989). The construction of the isoform mRAR-γ2 expression vector has been reported (Kastner et al., 1990).

The GALA/RAR-γ(A/B) chimera was constructed by replacing the human estrogen receptor (hER) exon 7 in the vector GALA-Exon7-F (Webster et al., 1989) with a 265-bp XhoI-KpnI fragment encoding amino acids 1-89 of mRAR-γ (A/B region). Amino acids in the linker between GALA (1-147) and the RAR-γ A/B region are IGRPPRA. The GALA/RAR-γ(EF) and (DEF) constructs were made similarly by replacing hER exon 7 with a 782-bp XhoI-KpnI fragment (encoding amino acids 201-458 of mRAR-γ) and a 917-bp XhoI-KpnI fragment (amino acids 156-458 of mRAR-γ), respectively. GALA/RAR-γ(DEF) and (DEF) chimeras also contain the amino acids IGRPPRA in the linker region. The mRAR-γ1 XhoI-KpnI cassettes that had been modified by two rounds of site directed mutagenesis to introduce XhoI and KpnI restriction sites at amino acids 553-595 of hER (F region) as a carboxylterminal antigenic tag against IGRPPRA in the linker region of the isoform mRAR-γ2 expression vector has been reported (Kastner et al., 1988; Krust et al., 1989; Zelent et al., 1989). The construction of the isoform GALA-RAR-γ(EF) and (DEF) chimeras also contain the amino acids IGRPPRA. The GALA-RAR-γ(EF) and (DEF) constructsweremade similarly by replacing hER exon 7 with a 782-bp XhoI-KpnI fragment (encoding amino acids 201-458 of mRAR-γ) and a 917-bp XhoI-KpnI fragment (amino acids 156-458 of mRAR-γ), respectively. GALA/RAR-γ(DEF) and (DEF) chimeras also contain the amino acids IGRPPRA in the linker region. The mRAR-γ1 XhoI-KpnI cassettes were obtained from mRAR-γ clones that had been modified by two rounds of site directed mutagenesis to introduce XhoI and KpnI restriction sites at selected positions. Each of the three chimeric constructs encodes amino acids 553-595 of hER (F region) as a carboxylterminal antigenic tag against which mAbs (AbF3) have been raised (Rochette-Egly et al., 1990).

Cell Culture and Transfection

COS-1 cells were grown in 9-cm-diam Petri dishes, in DMEM, containing 5% FCS, 50 U penicillin, 400 µg gentamycin, and 100 µg streptomycin per milliliter. Cells were transfected by using the calcium phosphate technique as previously described (Brand et al., 1988). Transfections included either the mouse RAR-γ1, γ2, α1, or β2 expression vectors (5 µg) and plasmid carrier DNA (Bluescript) to adjust the total DNA quantity to 20 µg.

Synthesis of Peptides, Preparation of Antisera and mAbs

The synthetic peptides SP15 (Al region of mouse or human RAR-γ1), SP14 (F region of all mRAR-γ isoforms), SP81 (D2 region of all human and mouse RAR-γ isoforms) and SP25 (F region of all hRAR-γ isoforms) (see Table I) deduced from the cDNA of human and mouse RAR-γ were synthesized in solid phase using Fmoc chemistry (model 431A peptide synthesizer, Applied Biosystems, Inc., Foster City, CA), verified by amino acid analysis (model 420A-920A-130A analyzer system; Applied Biosystems, Inc.) and coupled to ovalbumin (Sigma Chemical Co., St. Louis, MO) through an additional NH2-terminal cysteine residue, using the bifunctional reagent deThé et al., 1990) as described in Nicholson et al. (1990). Nuclear proteins were obtained by SDS-PAGE (10% polyacrylamide), electrophoresed on a 20% SDS-polyacrylamide gel, transferred onto a nitrocellulose (NC) filter as described (Gaub et al., 1989) and immunoprobed as follows. The NC filters were “blocked” in PBS-3% nonfat powdered milk, and then incubated for 2 h at 37°C with rabbit polyclonal or mouse monoclonal antibodies at the required dilution in PBS. After extensive washing in PBS containing 0.05% Tween 20 and washing in PBS-0.3% nonfat powdered milk, the filters were incubated for 90 min at 20°C with either 125I-labeled Protein A or 125I-labeled goat anti-mouse immunoglobulins (Amersham International). After extensive washing with PBS/Tween 20, the filters were dried and autoradiographed. When monovalent, alkaline phosphatase-coupled immunoglobulins (goat anti-rabbit or mouse immunoglobulins, Jackson Immunoresearch, West Grove, PA) were used and staining was performed by using the NBT/BCIP substrate kit (Pierce Chemical Co., Rockford, IL).

Gel retardation assay

Mobility shift assays were performed as described by Aigner and Revzin (1981) using the wild-type and mutated double-stranded oligodeoxynucleotides (RARE-β and RARE-βm, respectively) corresponding to the RARE of the RAR-β gene (de Thé et al., 1990) as described in Nicholson et al. (1990). Nuclear extract, usually 5 µg protein, was incubated in a 20-µl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 4 µg poly(dI-dC), and 0.2 ng (20,000 cpm) double-stranded [32P]5'-end-labeled synthetic RARE oligodeoxynucleotide and, when requested, 1 µl of ascite fluid (diluted one-third). Poly(dI-dC) and nuclear extract were first incubated at 4°C for 15 min before adding the labeled oligodeoxynucleotide. After a further 15-min incubation on ice, the antibodies were added when requested, and the mixture was maintained on ice for 15 min before loading the gel. Free DNA and RNA protein complexes were resolved on a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris-base, 45 mM boric acid, 2 mM EDTA).

Immunoprecipitations

The cell extracts (50 µg protein) were first preabsorbed with non-immune serum or control ascite fluid in a 1 ml final volume of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100 (buffer C) with constant agitation.
at 4°C for 1 hr. Then Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) were added (100 µl of a 50% vol/vol slurry in buffer C) for a further one hour incubation. The "absorbed" extract, which was recovered in the supernatant after pelleting by centrifugation, was incubated with 1 µg ml⁻¹ of rabbit anti-mouse IgG Fraction (1.8 µg, Jackson ImmunoResearch) was required as a bridge. Protein A-Sepharose beads were then added for 1 hr at 4°C. After centrifugation, the pellet was washed four times with buffer C. Antigen-antibody complexes were eluted by incubation at 10°C for 10 min in 50 µl of electrophoresis sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, and 0.001% bromophenol blue). Immunoblotting was then performed as described above.

Alkaline Phosphatase Treatment of Immunoprecipitates

Immunoprecipitates were suspended in 100 µl phosphate reaction mixture containing 100 mM Tris-HCl buffer (pH 9.8), 1 mM MgCl₂, 0.1 mM ZnCl₂, PIC, and 20 U of calf intestinal alkaline phosphatase (Boehringer, Mannheim, Germany). Sodium phosphate 10 mM was included as indicated. Incubation was performed at 37°C for 3 h followed by centrifugation, washing, addition of electrophoresis sample buffer, heating, electrophoresis, and immunoblotting.

Phosphate Labeling

24-h transfected COS-1 cells were first starved overnight in phosphate-deficient DMEM, and then labeled with [³²P] (1 MCl/2 ml, ~2.10⁶ cells) for 4 h at 37°C. Cell monolayers were washed six times in ice-cold PBS and lysed by five successive freeze/thawings (at ~80°C) and thawings in buffer A. After centrifugation at 8,000 g for 20 min at 4°C, the supernatant was subjected to immunoprecipitation as described above. Proteins from the immune complexes were eluted, separated by SDS-PAGE electrophoresis, and electrotransferred to NC filters. The phosphorylated proteins were visualized by autoradiography. Proteins were identified as RAR-γ by incubation of the same filter with specific antibodies followed by an alkaline phosphatase-labeled second antibody as described above.

Results

(A) Preparation of Polyclonal and Monoclonal Antibodies against Synthetic Peptides Specific to Human and Mouse RAR-γ Isoforms

RAR-γ1 and γ2 isoforms, which differ from each other only in their NH₂-terminal A regions (A1 for RAR-γ1 and A2 for RAR-γ2) are highly conserved between mouse and human, both in their length which is identical, and in their amino acid sequences which are very similar with the exception of the very COOH-terminal region (Krust et al., 1989; Kastner et al., 1990; see also Table I). The main differences between RAR-γ isoforms and RAR-α and β isoforms are located in the NH₂-terminal A region, central D2 region, and carboxy-terminal F region (Zelent et al., 1989; Krust et al., 1989; Kastner et al., 1990). Thus, to obtain specific antibodies corresponding to known epitopes, we selected potential immunogenic amino acid sequences which were either specific to all RAR-γ isoforms (regions D2 and F) or unique to RAR-γ1 (region A1) (see Table I). Two of these peptides (SP15 and SP81, corresponding to regions A1 and D2, respectively) are fully conserved between human and mouse, whereas the two others (SP25 and SP14, corresponding both to region F) diverge by three or four amino acids.

The four peptides were antigenic in mice and resulted in the production of specific hybridomas. Based on the intensity of the reaction obtained by immunoblotting and immunoprecipitation, we selected one clone corresponding to each peptide: SP15 (Abγ1(A1)), SP14 (Ab2γ(mF)), SP25 (Ab4γ(hF)), and SP81 (Ab5γ(D2)). Each clone recognized specifically its cognate, but not other peptides, as checked by ELISA (data not shown). All four antibodies were identified as IgG κ kappa. Peptides SP14, SP15, and SP25 resulted also in the production of polyclonal antibodies in rabbits, but only the polyclonal antibody preparation against SP14 (RPγ(mF)), which gave the strongest reaction was further studied here. All these antisera were tested by immunocytochemistry for their ability to reveal the proteins generated by transfecting the expression vector RAR-γ in COS-I cells. As previously described for RAR-α and RAR-β (Gaub et al., 1989), an intense nuclear staining was observed when RAR-γ-transfected cells were treated with RPeγ(mF) (Fig. 1 A), Abγ1 (Al) (Fig. 1 D), Ab2γ (mF) (Fig. 1 F), and Ab5γ (D2) (Fig. 1 I). No staining was observed when the cells were treated with either nonreactive serum (Fig. 1 C) or nonreactive ascites (Fig. 1 G), or transfected with either mRAR-α (Fig. 1 E) or mRAR-β2 (Fig. 1 F).

(B) Specific Detection of Cloned Human and Mouse RAR-γ Proteins by Immunoblotting, Immunoprecipitation, and Gel Shift Assay

(i) Immunoblotting. The monoclonal antibodies as well as the rabbit polyclonal antisera were tested for their ability to

Table I. Amino Acid Sequence (Single Letter Code) of the Synthetic Peptides Used to Generate RAR-γ Antibodies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP15</td>
<td>mouse: FEHLSPSRFLGL</td>
<td>RAR-γ1</td>
</tr>
<tr>
<td></td>
<td>human: 39------------50</td>
<td></td>
</tr>
<tr>
<td>SP81</td>
<td>mouse: KEEGSPDSYELS</td>
<td>RAR-γ2</td>
</tr>
<tr>
<td></td>
<td>human: 172---------183</td>
<td></td>
</tr>
<tr>
<td>SP14</td>
<td>mouse: SSEDAPGGKQKGSQ</td>
<td>RAR-γ1</td>
</tr>
<tr>
<td></td>
<td>human: ------V-------G-LK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>436</td>
<td>451</td>
</tr>
<tr>
<td>SP25</td>
<td>human: QPGFHPNASEDEV</td>
<td>RAR-γ2</td>
</tr>
<tr>
<td></td>
<td>428</td>
<td>441</td>
</tr>
</tbody>
</table>

RAR-γ1 and RAR-γ2 (458 and 447 amino acids long proteins, respectively; same length in mouse and human) are schematically represented with their six regions designated A through F. RAR-γ1 and RAR-γ2 differ from each other only in their NH₂-terminal A region (A1 for RAR-γ1 and A2 for RAR-γ2). The amino acid sequence (single letter code) of the synthetic peptides used to generate RAR-γ antibodies is represented. The numbers flanking the peptide sequences correspond to the portion of the respective amino acid residues in the sequence of RAR-γ isoforms. Amino acids differing between mouse and human RAR-γ are indicated.

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reveal specifically on Western blots the cloned mouse or human RAR-γ proteins produced by transfected COS-1 cells (see Materials and Methods). Whole cell extracts (WCE) of COS-1 cells transfected with vectors expressing either the human or mouse RAR-γ1 isoform were fractionated by SDS-PAGE and electroblotted onto nitrocellulose (NC) filters. After incubation of the filters with the specific monoclonal antibodies or the rabbit antisera, antibody-antigen complexes were revealed by using 125I-anti-mouse immunoglobulins or 125I-Protein A respectively (Fig. 2 A).

In extracts of COS-1 cells transfected with mRAR-γ1 expression vector, the mAbs Ab1γ1(A1), Ab5γ(D2), and Ab2γ(mF), as well as the SPI4 rabbit antiserum RPγ(mF), resulted in a specific, strongly labeled signal with an apparent molecular mass of ≈51 kD (Fig. 2 A, lanes 2, 5, 8, and 14), which is in excellent agreement with the cDNA-deduced molecular mass of the mRAR-γ1 protein (M, = 50,347; Krust et al., 1989). It must be noted that a specific additional signal with a lower apparent molecular mass and of variable intensity depending on the cell extract was detected with the monoclonal Ab1γ1(A1) (Fig. 2 A, lane 2; see also Fig. 3 A, lane 7). Similarly, with monoclonal Ab2γ(mF) and polyclonal RPγ(mF), a specific additional minor signal with a higher apparent molecular weight (and variable intensity) was seen (Fig. 2 A, lanes 8 and 14; see also Fig. 3 A, lanes 1 and 13). No labeling was detectable with the mAb Ab4γ(hF) (data not shown). In hRAR-γ1-transfected COS-1 cells extracts, a similar specific 51-kD signal was also revealed by Western blotting with Ab1γ1(A1), Ab2γ(mF), and Ab5γ(D2) (data not shown), as well as with Ab4γ(hF) (Fig. 2 A, lane 11). However, the RPγ(mF) antiserum did not recognize the human cloned receptor (data not shown).

When the ascites fluids and the antisera were depleted from the specific antibodies as described in Materials and Methods, all of the above specific signals were no longer seen (Fig. 2 A, lanes 3, 6, 9, 12, and 15). Similar competition experiments using ovalbumin alone did not affect the intensity of the specific signals (data not shown). No specific labeling was observed on Western blots performed with extracts of untransfected COS-1 cells (Fig. 2 A, lanes 1, 4, 7, 10, and 13), suggesting a very low level of expression of RAR-γ1 protein in these cells. Furthermore, no cross-reactions were seen with the same antibodies using extracts from COS-1 cells transfected with either RAR-α1 or RAR-β2, (data not shown), as confirmed by immunocytochemistry (Fig. 1, E and F), indicating that the present antibodies are specific for the RAR-γ1 protein. However, as expected, Ab5γ(D2), Ab2γ(mF), and RPγ(mF) reacted also specifically with extracts from mRAR-γ2 transfected COS-1 cells and revealed a protein with an apparent molecular weight of ≈48 kD (Fig. 2 A, lanes 19, 21, and 23). In some instances, and for unknown reasons, this 48-kD species was strongly decreased in favor of a specifically reacting protein with an apparent molecular weight of ≈45 kD (see for instance Fig. 2 B, lanes 16 and 20; and Fig. 3 C, lanes 2 and 7). In contrast, the mRAR-γ2 protein present in these extracts was not recognized by Ab1γ1(A1), in agreement with the presence of a different A region (A2) in the mRAR-γ2 isoform (see above) (Fig. 2 A, lane 17).

These results demonstrate that the mAbs Ab2γ(mF) and Ab5γ(D2) recognize specifically the corresponding epitopes present in both human and mouse RAR-γ proteins, whereas the Ab4γ(hF) antibody recognizes specifically the corresponding epitope present in human RAR-γ isoforms. Conversely, the polyclonal antiserum RPγ(mF) recognizes only the corresponding epitope present in mouse RAR-γ isoforms. These results show also that the monoclonal antibody Ab1γ1(A1) reacts specifically with the corresponding epitope which is present in human and mouse RAR-γ1 isoform, but not in human and mouse RAR-γ2 isoform.

(b) Immunoprecipitation. The three mAbs (Ab1γ1(A1), Ab2γ(mF), and Ab5γ(D2)) as well as RPγ(mF) also specifically immunoprecipitated mRAR-γ1 protein from whole cell extracts of mRAR-γ1-transfected COS-1 cells, as shown by subsequent Western blotting (Fig. 2 B, lanes 9-12). As expected (see above), no specific signal was seen when extracts from mRAR-γ2-transfected cells were immunoprecipitated with Ab1γ1(A1) (Fig. 2 B, lane 17), whereas signals were observed when using the same extracts and either Ab5γ(D2)
Figure 2. (A) Characterization of monoclonal and polyclonal antibodies by Western blotting. COS-1 cells extracts were either from control untransfected COS-1 cells (lanes 1, 4, 7, 10, and 13) or from COS-1 cells transfected with mRARγ-1 (lanes 2, 3, 5, 6, 8, 9, 14, 15, 16, 18, 20, and 22), hRAR-γ-1 (lanes 11 and 12) or mRARγ-2 (lanes 17, 19, 21, and 23) expression vectors. The extracts were fractionated by SDS-PAGE, electrotransferred onto NC filters and then immunoprobed with the mAbs Ably1(A1) (lanes 1-3, 16, and 17), Ab5γ(D2) (lanes 4-6, 18, and 19), Ab2γ(mF) (lanes 7-9, 20, and 21), Ab4γ(hF) (lanes 10-12) or the rabbit polyclonal antibodies RPy(mF) (lanes 13-15, 22, and 23), without (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, and 16-23) or with previous antibody depletion (lanes 3, 6, 9, 12, and 15) as described in Materials and Methods. The position of the prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) is indicated in kilodaltons. (B) Characterization of monoclonal and polyclonal antibodies by immunoprecipitation. Extracts from either pSG5 (lanes 2-7), mRARγ-1 (lanes 9-14), or mRARγ-2 (lanes 17-19) transfected COS-1 cells were immunoprecipitated (as described in Materials and Methods) with the following antibodies: RPy(mF), lanes 4 and 9; Ably1(A1), lanes 5, 10, and 17; Ab5γ(D2), lanes 6, 11, and 18; Ab2γ(mF), lanes 7, 12, and 19; nonreactive rabbit serum (NRS), lanes 2 and 13; nonreactive ascite (NRA), lanes 3 and 14. Antigen–antibody complexes bound to Protein A Sepharose beads were eluted, fractionated by SDS-PAGE, and electrotransferred to NC filters. The immunoprecipitated material was immunoprobed by incubation of the filters with RPy(mF) and 125I-Protein A. As positive controls, extracts (10 μg protein) of mRARγ-1 (COS-γ1, lanes 1, 8, 15, and 21) or mRARγ-2, lanes 16 and 20) transfected COS-1 cells were directly loaded on the gel without prior immunoprecipitation, and then immunoprobed. (C) Characterization of mAbs by their effects on the DNA–protein complexes formed in vitro with the RARE of the RAR-β2 promoter using gel retardation/shift assay. Gel retardation reactions were carried out with 5 μg of extracts from COS-1 cells transfected with either mRARγ-1 (lanes 1-7), mRARα-1 (lanes 8-13) or mRARβ-2 (lanes 14-17) expression vectors. Arrow 1 indicates the specific complexes formed with the RARE-β probe. Arrow 2 indicates the shifted complex formed in the presence of the monoclonal antibodies: Ably1(A1), lane 3; Ab2γ(mF), lanes 4, 10 and 16; Ab5γ(D2), lane 5; Ab9α(hF), lanes 6 and 11; Ab7β2 (A1): lanes 7, 12 and 17; Non Reactive Ascite (NRA), lane 13. The monoclonal antibodies Ab9α(hF) and Ab7β2(A1) have been raised against synthetic peptides corresponding to amino acid stretches of the F region of RAR-α1, and of the A1 region of RAR-β2, respectively (manuscripts in preparation).
Figure 3. Characterization of RAR-γ isoforms in F9 cells and mouse embryos. (A) Immunoblotting. Nuclear extracts (70 µg protein) of F9 cells (lanes 1-15) and mouse embryos (lanes 16-22) were fractionated by SDS-PAGE, electrotransferred to NC filters and immunoblotted with: RPy(mF), lanes 1-9, 16-20; Abγ(A1), lanes 7, 9; Ab5γ(D2), lanes 10-12; Ab2γ(mF), lanes 13-15. The incubations were also performed with antibody-depleted RPy(mF) lanes 4-6, 21 and 22. F9 cells were tested either with (lanes 3, 6, 9, 12, and 15) or without (lanes 2, 5, 8, 11, and 14) a 24-h retinoic acid treatment. Mouse embryos were tested at 11.5 d (lane 17), 13.5 d (lanes 18 and 22), 14.5 d (lane 19) and 17.5 d (lane 20). As positive controls, extracts of mRAR-γ1-transfected COS-1 cells (COS-γ1) were run in parallel (lanes 1, 4, 7, 10, 13, 16, and 21). (B) Characterization of RAR-γ in mouse embryos by immunoprecipitation. Nuclear extracts (1 mg protein)
or Ab2γ(mF) (Fig. 2 B, lanes 18 and 19). Also, as expected 
RPγ(mF) did not immunoprecipitate hRAR-γ1 from extracts 
of hRAR-γ1-transfected cells, whereas Ab4γ(hF) did it but 
with a lower efficiency than Ab1γ(Al), Ab5γ(D2), or 
Ab2γ(mF) (data not shown). In all cases the signals were 
specific, since they were not observed when immunoprecipita-
tion was performed with preimmune nonreactive serum 
(NRS) or a control nonreactive ascite fluid (NRA) (Fig. 2 B, 
lanes 13 and 14) or with cell extracts transfected with the par-
ent expression vector pSG5 (Fig. 2 B, lanes 4–7). Further-
more, the signals disappeared specifically when the Western 
blotting step was performed with an antibody-depleted 
ascite fluid or serum (data not shown). A minor signal cor-
responding to the immunoprecipitating rabbit immunoglobulins 
was occasionally revealed (data not shown, and Fig. 2 B, lane 4).

(iii) Gel Shift Assay. To confirm the specificity of the 
present antibodies for the RAR-γ isoforms, gel shift/retarda-
tion assays were performed using a 32P-labeled oligodeoxy-
nucleotide (RARE-β, see Materials and Methods) contain-
ing the RA response element (RARE) of the RAR-β 
promoter (de Thé et al., 1990; Suvov et al., 1990; Nicholson 
et al., 1990; Zelent et al., 1991; Vasios et al., 1991). With 
extracts of COS-1 cells transfected with mRAR-γ1, a specific 
complex was obtained (arrow 1 in Fig. 2 C, lane 2) which 
disappeared when the oligonucleotide was mutated (RARE-
βm, see Materials and Methods) (Fig. 2 C, lane 1). The 
above complex was shifted to a more slowly migrating 
species (arrow 2 in Fig. 2 C) after the addition of the monoocl-
al antibodies Ab1γ(Al) (Fig. 2 C, lanes 3 and 4). However, Ab5γ(D2) was less effective in inducing such a shift (Fig. 2 C, lane 5). Similarly, the addition of ei-
ther Ab1γ(Al) (see Nicholson et al., 1990) or Ab4γ(hF) 
(see Vasios et al., 1991) resulted in a shift of the probe–re-
ceptor complex obtained with extracts of hRAR-γ1-trans-
fected cells. As expected, the probe–receptor complexes 
formed with mRAR-γ2-transfected cells were clearly shifted 
with Ab2γ(mF) and to a less extent with Ab5γ(D2), whereas 
no shift was observed with Ab1γ(Al) (data not shown). In 
contrast, mAbs specifically directed against either mRAR-
αl [Ab9α(hF)] or mRAR-β (Ab7β2(Al)) did not induce any 
shift of the probe–RAR complex obtained with COS-1 cells 
expressing mRAR-γ1 (Fig. 2 C, lanes 6 and 7). Furthermore 
one of the mAbs raised against mRAR-γ1 led to a shift of 
the probe–RAR complex obtained with COS-1 cells express-
ing either mRAR-αl or mRAR-β2 (Fig. 2 C, lanes 10 and 
16, and data not shown), thus confirming that they are spe-
cific for RAR-γ isoforms.

(C) Detection of RAR-γ Isoforms in F9 Embryonal 
Carcinoma Cells and Mouse Embryos

We investigated whether all of the RAR-γ antibodies charac-
terized above could detect the presence of RAR-γ isoforms 
in mouse F9 embryonal carcinoma cells and mouse embryos. 

mRAR-γ1 and mRAR-γ2 messenger RNAs have indeed been 
found in F9 cells and in mouse embryos at various stages of 
development (Zelent et al., 1989; Kastner et al., 1990). The 
possible presence of mRAR-γ isoforms was first investigated 
by Western blotting using nuclear extracts from either F9 
cells (treated or not with RA) or mouse embryos. No signal 
was detected when the mAbs Ab1γ(Al), Ab5γ(D2), and 
Ab2γ(mF) were used (Fig. 3 A, lanes 7–15 and data not 
shown). However, with the RPγ(mF) antiserum a signal cor-
responding to a protein with an apparent molecular mass 
of 85 kD was detected, instead of the expected 51-kD cloned 
RAR-γ molecule (Fig. 3 A, lanes 2, 3, and 17–20, arrow).

This signal, which was specific since it disappeared after an-
tibody depletion of the antiserum (Fig. 3 A, lanes 5, 6, and 
22), may correspond to a 85-kD protein bearing a similar 
cross-reacting epitope(s). The lack of signals with the mono-
clonal antibodies suggested that the epitopes recognized by 
these antibodies could be modified posttranslationally in F9 
cells and mouse embryos, and/or that the RAR-γ proteins 
may be synthesized in amounts too low to be detectable by 
Western blotting.

We thus performed immunoprecipitation experiments 
using the same cell and embryo extracts. A protein with the 
expected RAR-γ1 molecular mass (51 kD) was revealed on 
Western blots using RPγ(mF) after immunoprecipitation of 
nuclear extracts of mouse embryos (14.5 d p.c.) with either 
Ab1γ(Al) (Fig. 3 B, lane 3), Ab2γ(mF) (Fig. 3 B, lane 4), 
Ab5γ(D2) (Fig. 3 B, lane 5). Note that, to be seen, these 
signals required that 1 mg of nuclear proteins was immuno-
precipitated. However, they were specific since they disap-
peared when the NC filter was revealed with antibody-de-
pleted RPγ(mF) (data not shown). Using F9 cell extracts, 
two signals (corresponding either to a molecular mass simi-
lar to that of RAR-γ2 [~48 kD] or to a lower one [~42 kD], 
were specifically immunoprecipitated from 1 mg of nuclear 
proteins with Ab2γ(mF) (Fig. 3 C, lane 4), Ab5γ(D2) (Fig. 3 
C, lane 5). However no signal was seen when F9 cell ex-
trates were immunoprecipitated with Ab1γ(Al) (Fig. 3 C, 
lane 3). The same pattern was observed whether the F9 
cells were treated or not for 24 h with RA (data not shown).

Moreover, the obtained signal was not increased when the three 
mAbs were added together (data not shown).

(D) Phosphorylation of Mouse RAR-γ1

Multiple electrophoretic species were seen for the mRAR-γ1 
protein made in COS-1 cells, and revealed with antibodies 
Ab1γ(Al), Ab2γ(mF), and RPγ(mF) (Fig. 2 A and 3 A), which 
suggests the possible occurrence of post-translational modifi-
cations. Protein phosphorylation often alters mobility
during SDS-PAGE. Thus Ab$_2$$_y$(mF) immunoprecipitates of mRAR-γ$_1$-transfected COS-1 cell extracts were treated with calf intestinal alkaline phosphatase (CIP) in the presence or absence of sodium phosphate, a phosphatase inhibitor. In the absence of inhibitor, CIP treatment increased the mobility of mRAR-γ$_1$, as compared to the untreated controls (Fig. 4, lanes 4 and 5). This effect was no longer visible after phosphatase treatment in the presence of inhibitor (Fig. 4, lane 6).

To confirm these results, we examined the phosphorylation state of RAR-γ$_1$ and the effect of retinoic acid treatment on phosphorylation. mRAR-γ$_1$-transfected COS-1 cells were labeled with $[^{32}P]$orthophosphate in the presence or absence of RA (10$^{-7}$ M) and the RAR-γ$_1$ proteins were immunoprecipitated with the specific mAbs Ab$_2$$_y$(mF). A phosphorylated protein with an apparent molecular mass of 51 kD and corresponding to RAR-γ$_1$ (as determined by immunoblotting on the same NC filters, using RP$_y$(mF) and alkaline phosphatase conjugated goat anti–rabbit antibody) was detected (Fig. 5A, lanes 2, 3, 6, and 7). No variation in the phosphorylation intensity was seen after 4 h of RA treatment (Fig. 5A, compare lanes 6 and 7). No phosphorylated protein was detectable in COS-1 cells which had been transfected with the parental expression vector pSG5 (Fig. 5A, lanes 4, 5, 8, and 9). These results indicate that the RAR-γ$_1$ protein can exist in a phosphorylated state.

To investigate which domain(s) of RAR-γ$_1$ are phosphorylated, we constructed three expression vectors encoding chimeric proteins, Gal4-RAR-γ$_1$ (A/B), Gal4-RAR-γ$_1$ (EF), and Gal4-RAR-γ$_1$ (DEF) in which the Gal4 (1-147) DNA binding domain is fused with either the A/B, EF, or DEF regions of mRAR-γ$_1$, respectively. These chimeric proteins also contained the F region of the estrogen receptor (ER) against which immunoprecipitating monoclonal antibodies (AbF3) have been raised (Rochette-Egly et al., 1990).

COS-1 cells were transfected, labeled with $[^{32}P]$ and the chimeric proteins were immunoprecipitated with the mAb AbF3. The expected chimeric proteins were revealed after electrophoresis by immunoblotting (Fig. 5B, lanes 1, 3, 5, and 7, arrows; Gal4-Exon(8) is a chimeric protein that contains the Gal4 DNA binding domain fused to the ER region F; see Webster et al., 1989). Autoradiography of the same NC filters revealed that the proteins encoded by the Gal4-RAR-γ$_1$(A/B) and Gal4-RAR-γ$_1$(DEF) expression vectors, were phosphorylated (Fig. 5B, lanes 4 and 8). The phosphorylation of GAL4-RAR-γ$_1$(DEF) was not affected by RA treatment, (data not shown). However, the protein encoded by the Gal4-RAR-γ$_1$(EF) expression vector was not labeled either in the presence or absence of RA (Fig. 5B, lane 6), suggesting that the D region, but not the EF region, might contain phosphorylation site(s). No $[^{32}P]$ labeling was associated with the Gal4-Exon(8) protein indicating that the Gal4-DNA binding domain as well as the F region of the estrogen receptor were not phosphorylated under these conditions (Fig. 5B, lane 2).

**Discussion**

In this study, we have described the production, characterization, and use of antipeptide antibodies that are directed against RAR-γ isoforms. Four monoclonal antibodies directed against the A1 region (Ab$_1$$_y$(A1)), the D2 region (Ab$_2$$_y$(D2)), and the F region (Ab$_2$$_y$(mF) and Ab$_4$$_y$(hF))

![Figure 4. Alkaline phosphatase treatment increases the electrophoretic mobility of mRAR-γ$_1$ protein. Extracts of mRAR-γ$_1$ transfected COS-1 cells were immunoprecipitated using the Ab$_2$$_y$(mF) mAbs (lanes 3-6) and the antigen–antibody complexes immobilized on Protein A-Sepharose beads were incubated with (lanes 5 and 6) or without (lane 4) calf intestinal alkaline phosphatase (CIP) in the absence (lanes 4 and 5) or presence (lane 6) of 10 mM sodium phosphate. The untreated (lane 3) and incubated (lanes 4-6) immunoprecipitates were then solubilized, subjected to electrophoresis, and electrotransferred to NC filters. The mRAR-γ$_1$ protein was identified by incubation of the filter with RP$_y$(mF) and [125$I$]Protein A. In parallel, extracts of COS-1 cells transfected with mRAR-γ$_1$ (lanes 2 and 7) or mRAR-γ$_2$ (lanes 1 and 8) expression vectors were run without prior immunoprecipitation.](https://jcb.rupress.org/issue/115/4/542)
Figure 5. Phosphorylation of mRAR-γ. (A) COS-1 cells transfected with either pSG5 (lanes 4, 5, 8, and 9) or mRAR-γ expression vector (lanes 2, 3, 6, and 7) were labeled with [32P] in the presence or absence of retinoic acid (see Materials and Methods) and were analyzed by immunoprecipitation with Ab5y(D2). After electrophoresis and electrotransfer to NC filters the immunoprecipitated phosphorylated proteins were visualized by autoradiography (lanes 6-9) and identified by incubation of the same NC filter with RPy(mF) followed by alkaline phosphatase-labeled antibodies (lanes 2-5). Extracts (10 μg protein) of mRAR-γ transfected COS-1 cells were run and electroblotted in parallel as a control (lane 1). (B) COS-1 cells were transfected with the following chimeric expression vectors: GAL4-Exon(8) (lanes 1 and 2), GAL4-RAR-γ(A/B) (lanes 3 and 4), GAL4-RAR-γ(EF) (lanes 5 and 6), GAL4-RAR-γ(DEF) (lanes 7 and 8). After labeling with [32P], the extracts were immunoprecipitated with the mAbs AbF3 (see Materials and Methods). The immunoprecipitates were eluted, subjected to electrophoresis and electrotransferred to NC filters. The phosphoproteins were analyzed by autoradiography (lanes 2, 4, 6, and 8) and identified by incubation of the same NC filter with AbF3 and revelation with alkaline phosphatase–labeled anti-mouse antibodies (lanes 1, 3, 5, and 7). The arrows indicate the position of the proteins produced by the chimeric expression vectors. Asterisks indicate contaminating immunoglobulins.
gamma(DEF) expression vector and not that encoded by Gal4-RAR-gamma(DEF) was phosphorylated, we cannot exclude the possibility that the EF region could in fact be phosphorylated in a D domain-dependent manner. Whether the DNA binding domain (region C) may also be phosphorylated remains to be investigated.

Although the precise location of the phosphorylated residues of RAR-gamma is unknown, we note that both the A/B and D regions correspond to the portion of the protein that contains most of serine residues which are in consensus sequences of known kinases, such as cAMP-dependent kinase (RSXS), proline directed kinase (SXSP), casein kinases I (EXXS) and II (SXXE, SXSP), and glycogen synthase kinase 3 (SXSSXXS) (see Kemp and Pearson, 1990 and Table II). Phosphorylation in the A/B domain has been also reported for the glucocorticoid (Hoeck and Groner, 1990; Bodwell et al., 1991) and thyroid hormone (Goldberg et al., 1988; Glineur et al., 1989) receptors. Moreover phosphorylation of serine residues has been observed in both the NH2-terminal region and the central D region (between the DNA and hormone binding domains) of the progesterone receptor (Denner et al., 1990a).

However, phosphorylation of RAR-gamma occurs irrespective of the presence of RA, in contrast to what has been found for the progesterone, glucocorticoid, and 1,25-dihydroxyvitamin D3 receptors whose phosphorylation increases in the presence of hormone (Denner et al., 1990b; Bodwell et al., 1991; Brown and Deluca, 1990). The possible effect of phosphorylation on the function of RAR-gamma is unknown. In this respect, we note that the role of phosphorylation on the function of other members of the nuclear receptor superfamily (see above for references) remains also to be discovered. Phosphorylation could affect the tertiary structure of RAR-gamma, which may result in the "unmasking" of a transcriptional activation function, as it was recently proposed in the case of the transcription factor CREB (Gonzalez et al., 1991). Phosphorylation may also control the rate of nuclear transport of RAR-gamma as it was shown in the case of SV40 T antigen whose nuclear localization signal (NLS) is flanked by a casein kinase II site (Rihs et al., 1991). We note in this respect that the D region of RAR-gamma contains casein kinase II sites as well as stretches of basic amino acids which may correspond to NLS. Site directed mutagenesis of the potential phosphorylation sites in RAR-gamma is obviously required to investigate this and other possibilities.

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References


Table II. Protein Kinase Phosphorylation Site Motifs

<table>
<thead>
<tr>
<th>Protein kinases</th>
<th>Possible phosphorylation recognition motifs</th>
</tr>
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<tbody>
<tr>
<td>In the A/B domain</td>
<td></td>
</tr>
<tr>
<td>cAMP-dependent kinase</td>
<td>34R GS*</td>
</tr>
<tr>
<td>Casein kinase I</td>
<td>40E MLS*</td>
</tr>
<tr>
<td></td>
<td>57E MAS*</td>
</tr>
<tr>
<td></td>
<td>64E TQG*</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>18S Gly</td>
</tr>
<tr>
<td></td>
<td>67S TSS</td>
</tr>
<tr>
<td></td>
<td>69S SE</td>
</tr>
<tr>
<td>Proline-dependent kinase</td>
<td>36S P</td>
</tr>
<tr>
<td></td>
<td>43S P</td>
</tr>
<tr>
<td></td>
<td>77S P</td>
</tr>
<tr>
<td></td>
<td>79S P</td>
</tr>
<tr>
<td>In the D domain</td>
<td></td>
</tr>
<tr>
<td>Casein kinase I</td>
<td>173E EG S*</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>176S PD S</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3</td>
<td>179S YEL S</td>
</tr>
<tr>
<td>Proline-dependent kinase</td>
<td>176S P</td>
</tr>
<tr>
<td></td>
<td>183S P</td>
</tr>
</tbody>
</table>

The localization of the possible phosphorylation recognition motifs for a number of protein kinases in the amino acid sequence of A/B and D regions of RAR-gamma (see Kemp and Pearson, 1990) is represented. The phosphate acceptor sequences are indicated with an asterisk. Where the specificity determinants for a protein kinase are known, determinant residues are underlined. Numbers indicate the position of the first amino acid in the putative recognition motif.

Glineur, C., M. Bailly, and J. Ghysdael. 1989. The c-erbA-encoded thyroid hormone receptor is phosphorylated in its amino terminal domain by casein kinase II. Oncogene. 4:1247–1254.


