Retinoic Acid Receptor γ:
Specific Immunodetection and Phosphorylation

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Abstract. 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(A1)), 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(A1)) 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 NH_2-terminal A/B region.

R ETINOIC 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 RA receptors (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

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1. *Abbreviations used in this paper:* NE, nuclear extracts; p.c., post-coitum; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; NC, nitrocellulose; WCE, whole cell extracts.

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-γ1 (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 GAL4/RAR-γ1(A/B) chimera was constructed by replacing the human estrogen receptor (hER) exon 7 in the vector GAL4-Exon7-F (Webster et al., 1989) with a 265-bp XhoI-KpnI fragment encoding amino acids 1-89 of mRAR-γ1 (A/B region). Amino acids in the linker between GAL4 (1-147) and the RAR-γ1 A/B region are IGRPPRA. The GAL4-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-γ1) and a 917-bp XhoI-KpnI fragment (amino acids 156-458 of mRAR-γ1), respectively. GAL4-RAR-γ(EF) and (DEF) chimeras also contain the amino acids IGRPPRA in the linker region. The mRAR-γ1 XhoI-KpnI cassettes were obtained from mRAR-γ1 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 carboxyl terminal 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, 500 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 per dish. F9 EC cells were grown and treated with retinoic acid (10^{-7} M) for 24 h where indicated.

Synthesis of Peptides, Preparation of Antisera and mAbs

The synthetic peptides SP15 (A1 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-γ1 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-extraterminal cysteine residue, using the bifunctional reagent MBS (Aldrich Chemical Co., Milwaukee, WI).

Rabbit immunization and antisera preparation have been previously described (Gaub et al., 1989). For mAbs preparation, 8-wk-old female Balb/c mice were injected intraperitoneally with 100 μg of coupled antigens. 4 d before the fusion, positive mice received a booster injection of antigen (100 μg), and then 10 μg (intravenous and intraperitoneal route) every day until spleen removal. The spleen cells were fused with Sp2/O-Ag14 myeloma cells essentially according to St. Groth and Scheidegger (1980). Culture supernatants were screened by ELISA using the unconjugated peptide as antigen. Positive cultures were then tested by immunofluorescence and Western blotting on RAR-γ1 cDNA-transfected COS-1 cells as described by Lutz et al. (1988). Hybridomas secreting antibodies recognizing specifically RAR-γ1 were cloned twice on soft agar. Each hybridoma was also adapted in serum-free medium SFRI-4 (Société française de Recherche et d'Investissement, Berganton, France). For ascite fluid production, 2 × 10⁶ cells were injected in pristane-primed Balb/c mice. Class and subclass determination was performed using an Isotyping Kit (Amersham International, Amer-

sham, UK). Both SFRI culture supernatants and ascite fluids were used as monoclonal antibody sources.

Preparation of Whole Cell and Nuclear Extracts from Cultured Cells and Mouse Embryos

Whole cell extracts (WCE) were prepared from confluent transfected cultures of COS-1 cells. Cells were washed with chilled PBS, scrapped and centrifuged. The pellet was homogenized at 4°C with a glass Dounce B homogenizer (20 pestle strokes) in 2 vol of 10 mM Tris-HCl pH 8, containing 20 mM sodium molybdate, 0.6 M KCl, 1.5 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (PIC; leupeptin, aprotinin, pepstatin, antitrypsin, and chymostatin at 0.5 μg/ml each). After centrifugation for 1 h at 105,000 g and 4°C, the supernatant was concentrated by ultrafiltration through Centricon 30 microconcentrators (Amicon Corp., Danvers, MA). Glycerol was added to 25% final concentration and the extracts were aliquoted and kept at −80°C. For the preparation of nuclear extracts (NE), the washed cells were first lysed at 4°C with a glass Dounce B homogenizer (15 strokes) in buffer A (20 mM Tris-HCl pH 8, 1 mM MgCl₂, 20 mM KCl, 1 mM DTT, 0.3 mM PMSF, PIC). After centrifugation for 5 min at 1,500 g at 4°C, the crude nuclear pellet was washed twice, resuspended in high-salt buffer B (same as buffer A but with 0.6 M KCl and 25% glycerol) and homogenized with Dounce B (20-30 strokes). Extraction of nuclear proteins was performed on ice under gentle vortexing. After centrifugation for 1 h at 105,000 g, the supernatant was concentrated by using microconcentrators (see above), aliquoted and frozen in liquid nitrogen.

Mouse embryos were collected at 11.5, 13.5, 14.5, and 17.5 d post-coitum (p.c.) and nuclear extracts were prepared, according to the same protocol except that the crude nuclear pellet was further purified in some cases by centrifugation on a 1.7 M sucrose cushion (30 min at 1,500 g) and was recovered at the interphase. Proteins were quantified by the method of Bradford (1976).

Immunoblotting

Proteins (10-70 μg) from either whole cell or nuclear extracts were fractionated by SDS-PAGE (10% polyacrylamide), electrotransferred 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 either 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 mentioned, alkaline phosphatase-coupled immunoglobulins (goat anti-rabbit or anti-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).

The specificity of the reaction was checked by depleting the antisera from the specific antibodies by incubation with nitrocellulose (NC) filter dotted with the coupled peptide (20 μg).

Gel Retardation Assay

Mobility shift assays were performed as in Garner 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 antibodies (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 reaction was maintained on ice for 15 min before loading the gel. Free DNA and DNA 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 the nonrelevant protein-IgG-protein A-sepharose complexes, was incubated with 3 μl of immune serum or ascite fluid for 1 h at 4°C. When using mAbs (IgG1 kappa) a further 1-h incubation with a rabbit anti-mouse IgG fraction (1.8 μg, Jackson ImmunoResearch) was required as a bridge. Protein A-Sepharose beads were then added for 1 h at 4°C. After centrifugation, the pellet was washed four times with buffer C. Antigen-antibody complexes were eluted by incubation at 100°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 phosphatase reaction mixture containing 100 mM Tris-HCl buffer (pH 9.8), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 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 [^{32}P](1 mCi/2 ml, ≈ 2.10^6 cells) for 4 h at 37°C. Cell monolayers were washed six times in ice-cold PBS and lysed by five successive freezings (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 mRAR-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 NH$_2$-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 NH$_2$-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 (Ab1γ1(A1)), SP14 (Ab2γ(mF)), SP25 (Ab4γ(hF)),

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

1	62	90	156	202			422	458	
A1	B	C	D1	D2	D3	E		F	RAR-γ1

1	51	79	145	191			411	447	
A2	B	C	D1	D2	D3	E		F	RAR-γ2

Peptide SP15 (A1 region) Ab1γ1 (A1)

mouse   FEHLSPSFRGLG
human   39 ---------- 50

Peptide SP81 (D2 region) Ab5γ (D2)

mouse   KEEGSPDSYELS
human   172 -------- 183

Peptide SP14 (mouse F region) Ab2γ (mF), RPγ(mF)

mouse   SSEDEAPGGQGKRGQS
human   ----- V ------ G-LK
          436   451

Peptide SP25 (human F region) Ab4γ (hF)

mouse   K ----- K ------ A
human   QPGPHPNASSEDEV
          428   441

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 NH$_2$-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 are 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.

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 IgG1 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-γ1 in COS-1 cells. As previously described for RAR-α and RAR-β (Gaub et al., 1989), an intense nuclear staining was observed when RAR-γ1-transfected cells were treated with RPγ (mF) (Fig. 1 A), Ab1γ1 (A1) (Fig. 1 D), Ab2γ (mF) (Fig. 1 H), 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-α1 (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
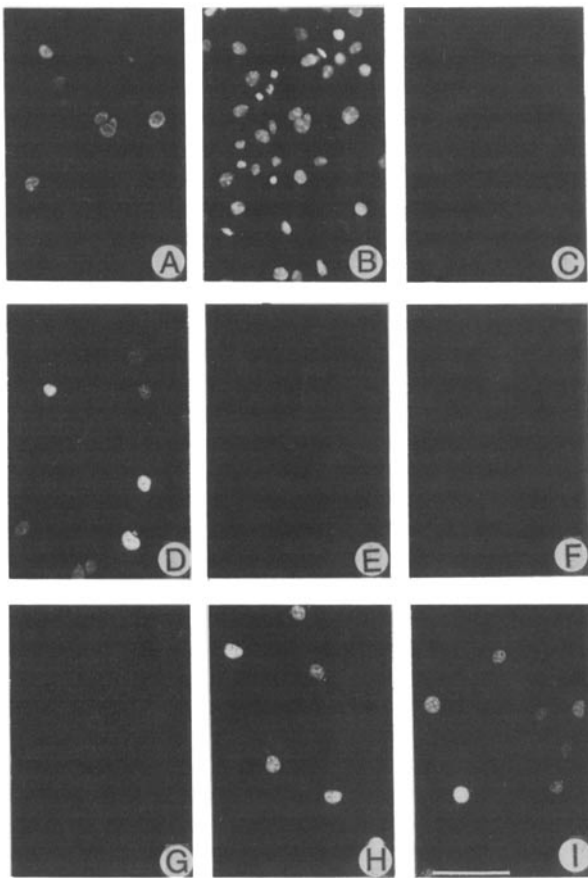
Figure 1. Immunocytochemistry of COS-1 cells transfected with mRAR-γ1 using either polyclonal or monoclonal anti-RAR-γ1 antibodies. (A) RPγ(mF); (D) Ab1γ1(A1); (H) Ab2γ(mF); (I) Ab5γ(D2). Transfected cells were fixed, permeabilized and incubated with the specific antibodies as previously described (Gaub et al., 1989). Then, Texas Red-labeled second antibodies were used as recommended by the manufacturer (Amersham International, Amersham, UK). Controls were performed with preimmune non-reactive serum (*NRS, C*) or nonreactive ascite fluid (*NRA, G*). Immunofluorescence was also performed with Ab1γ1(A1) on mRAR-α1 (*E*) and mRAR-β2 (*F*) transfected-COS-1 cells. Nuclei were stained with Hoechst reagent (*B*). Intense nuclear staining was observed with all antibodies (*A, D, H,* and *I*). Bar, 25 μm. ×400.

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 ^{125}I-anti–mouse immunoglobulins or ^{125}I-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 SP14 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_r = 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 ascite 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.

(ii) 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)
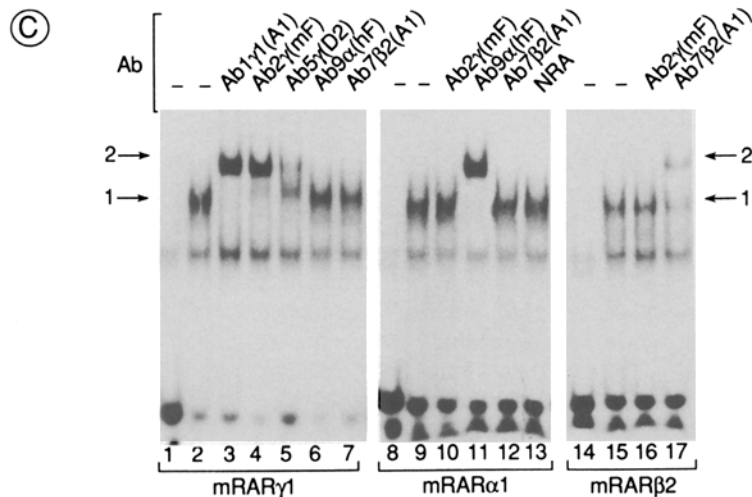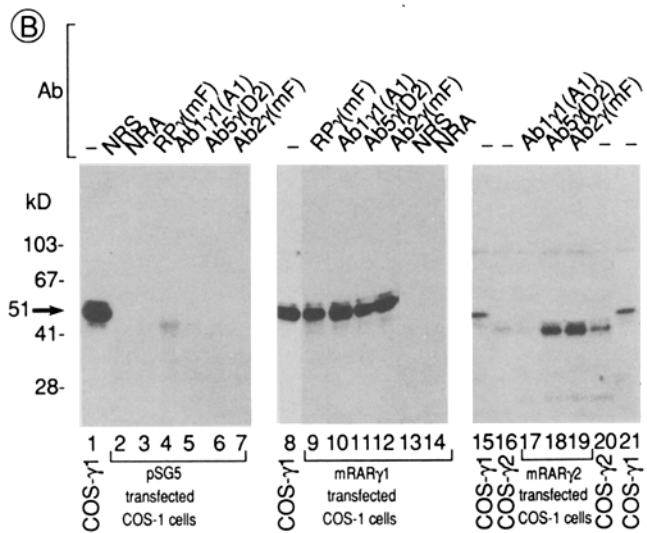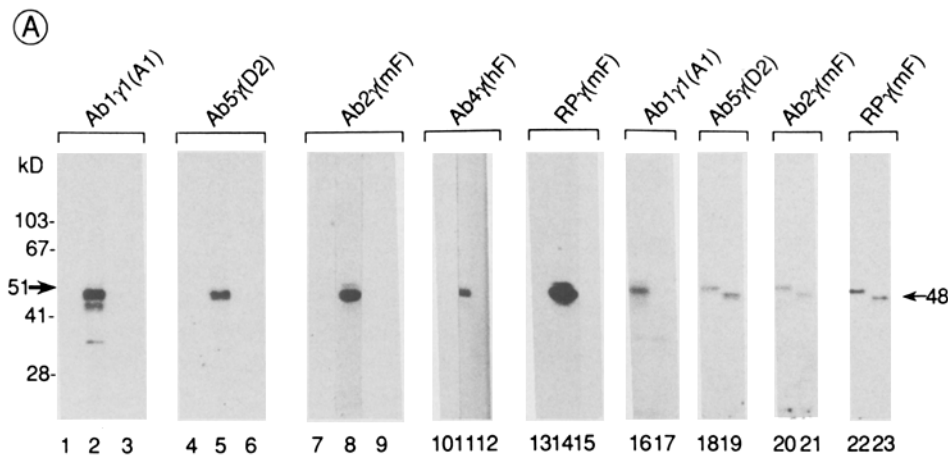
Figure 2. (*A*) Characterization of monoclonal and polyclonal antibodies by Western blotting. COS-1 cells extracts were either from control untransfected COS-1 cells (lanes *1, 4, 7, 10,* and *13*) or from COS-1 cells transfected with mRARγ-1 (lanes *2, 3, 5, 6, 8, 9, 14, 15, 16, 18, 20,* and *22*), hRAR-γ1 (lanes *11* and *12*) or mRAR-γ2 (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 Ab1γ1(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 RPγ-(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*), mRAR-γ1 (lanes *9–14*), or mRAR-γ2 (lanes *17–19*) transfected COS-1 cells were immunoprecipitated (as described in Materials and Methods) with the following antibodies: RPγ(mF), lanes *4* and *9*; Ab1γ1(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*; non-reactive 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 RPγ(mF) and ^{125}I-Protein A. As positive controls, extracts (10 μg protein) of mRAR-γ1 (COS-γ1, lanes *1, 8, 15,* and *21*) or mRAR-γ2, 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 mRAR-γ1 (lanes *1–7*), mRAR-α1 (lanes *8–13*) or mRAR-β2 (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: Ab1γ1(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).
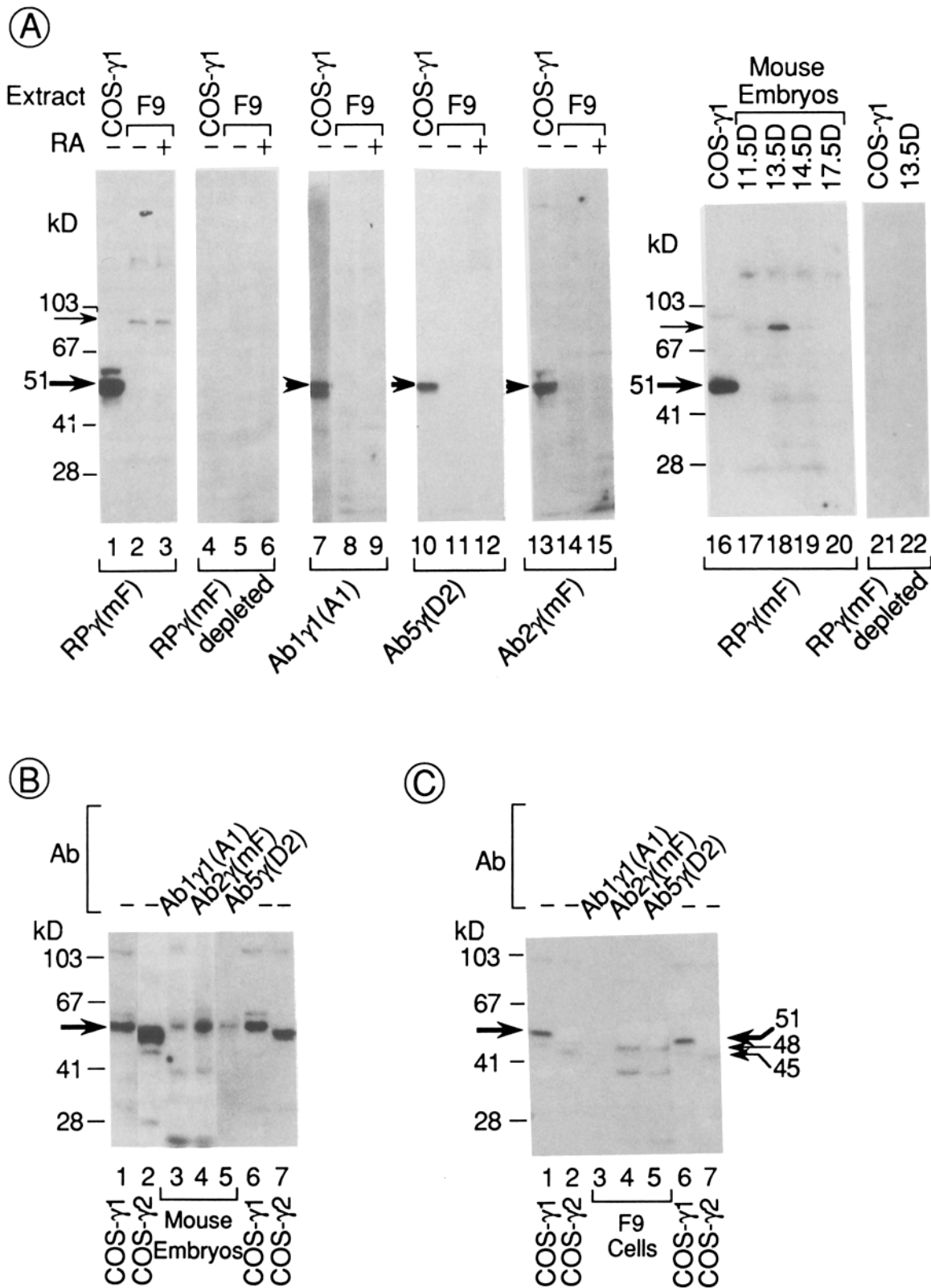
Figure 3. Characterization of RAR-γ isoforms in F9 cells and mouse embryos. (*A*) Immunoblotting. Nuclear extracts (70 μg protein) of F9 cells (lanes *1–15*) and mouse embryos (lanes *16–22*) were fractionated by SDS-PAGE, electrotransferred to NC filters and immunoprobed with: RPγ(mF), lanes *1–3, 16–20*; Ab1γ1(A1), lanes *7–9*; Ab5γ(D2), lanes *10–12*; Ab2γ(mF), lanes *13–15*. The incubations were also performed with antibody-depleted RPγ(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 Ab1γ1(A1), Ab5γ(D2), or Ab2γ(mF) (data not shown). In all cases the signals were specific, since they were not observed when immunoprecipitation 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 parental expression vector pSG5 (Fig. 2 B, lanes 4–7). Furthermore, 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 corresponding 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/retardation assays were performed using a ^{32}P-labeled oligodeoxynucleotide (RARE-β, see Materials and Methods) containing the RA response element (RARE) of the RAR-β2 promoter (de Thé et al., 1990; Sucov 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 monoclonal antibodies Ab1γ1(A1) and Ab2γ(mF) (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 either Ab1γ1(A1) (see Nicholson et al., 1990) or Ab4γ(hF) (see Vasios et al., 1991) resulted in a shift of the probe–receptor complex obtained with extracts of hRAR-γ1-transfected 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 Ab1γ1(A1) (data not shown). In contrast, mAbs specifically directed against either mRAR-α1 [Ab9α(hF)] or mRAR-β2 [Ab7β2(A1)] 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 none of the mAbs raised against mRAR-γ1 led to a shift of the probe-RAR complex obtained with COS-1 cells expressing either mRAR-α1 or mRAR-β2 (Fig. 2 C, lanes 10 and 16, and data not shown), thus confirming that they are specific 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 Ab1γ1(A1), 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 corresponding to a protein with an apparent molecular mass of 85 kD was detected, instead of the expected 51-kD cloned RAR-γ1 molecule (Fig. 3 A, lanes 2, 3, and 17–20, arrow). This signal, which was specific since it disappeared after antibody 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 monoclonal 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 Ab1γ1(A1) (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 immunoprecipitated. However, they were specific since they disappeared when the NC filter was revealed with antibody-depleted RPγ(mF) (data not shown). Using F9 cell extracts, two signals (corresponding either to a molecular mass similar 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 extracts were immunoprecipitated with Ab1γ1(A1) (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 Ab1γ1(A1), Ab2γ(mF), and RPγ(mF) (Fig. 2 A and 3 A), which suggests the possible occurrence of post-translational modifications. Protein phosphorylation often alters mobility

of 14.5-d mouse embryos were immunoprecipitated with the mAbs Ab1γ1(A1) (lane 3), Ab2γ(mF) (lane 4), or Ab5γ(D2) (lane 5). Antigen–antibody complexes bound to Protein A Sepharose beads were eluted, fractionated by SDS-PAGE, and electrotransferred to NC filters. The immunoprecipitated mRAR-γ proteins were immunoprobed by incubation of the filters with RPγ(mF) and [^{125}I]Protein A. As positive controls, extracts (10 μg protein) of mRAR-γ1 (lanes 1 and 6) and mRAR-γ2 (lanes 2 and 7) transfected COS-1 cells were directly loaded on the gel without prior immunoprecipitation and then immunoprobed. The arrow indicates the position of mRAR-γ1. (C) Characterization of RAR-γ in undifferentiated F9 cells by immunoprecipitation. Nuclear extracts (1 mg protein) of F9 cells were immunoprecipitated with the mAbs Ab1γ1(A1) (lane 3), Ab2γ(mF) (lane 4), and Ab5γ(D2) (lane 5). The immunoprecipitated RAR-γ proteins were immunoprobed as described in B with RPγ(mF). Extracts (10 μg protein) of mRAR-γ1 (lanes 1 and 6) or mRAR-γ2- (lanes 2 and 7) transfected COS-1 cells were directly loaded on the gel, as positive controls. The position of the mRAR-γ1 and mRAR-γ2 controls are indicated by thick and thin arrows respectively.

during SDS-PAGE. Thus Ab2γ(mF) immunoprecipitates of mRAR-γ1-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-γ1, 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-γ1 and the effect of retinoic acid treatment on phosphorylation. mRAR-γ1-transfected COS-1 cells were labeled with [^{32}P]orthophosphate in the presence or absence of RA (10^{-7} M) and the RAR-γ1 proteins were immunoprecipitated with the specific mAbs Ab2γ(mF). A phosphorylated protein with an apparent molecular mass of 51 kD and corresponding to RAR-γ1 (as determined by immunoblotting on the same NC filters, using RPγ(mF) and alkaline phosphatase conjugated goat anti–rabbit antibody) was detected (Fig. 5 A, lanes 2, 3, 6, and 7). No variation in the phosphorylation intensity was seen after 4 h of RA treatment (Fig. 5 A, 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. 5 A, lanes 4, 5, 8, and 9). These results indicate that the RAR-γ1 protein can exist in a phosphorylated state.

To investigate which domain(s) of RAR-γ1 are phosphorylated, we constructed three expression vectors encoding chimeric proteins, Gal4-RAR-γ1 (A/B), Gal4-RAR-γ1 (EF), and Gal4-RAR-γ1 (DEF) in which the Gal4 (1-147) DNA binding domain is fused with either the A/B, EF, or DEF regions of mRAR-γ1, 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. 5 B, 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-γ1(A/B) and Gal4-RAR-γ1(DEF) expression vectors, were phosphorylated (Fig. 5 B, lanes 4 and 8). The phosphorylation of GAL4-RAR-γ1 (DEF) was not affected by RA treatment, (data not shown). However, the protein encoded by the Gal4-RAR-γ1(EF) expression vector was not labeled either in the presence or absence of RA (Fig. 5 B, 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. 5 B, 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 (Ab1γ1(A1)), the D2 region (Ab5γ(D2)), and the F region (Ab2γ(mF) and Ab4γ(hF))
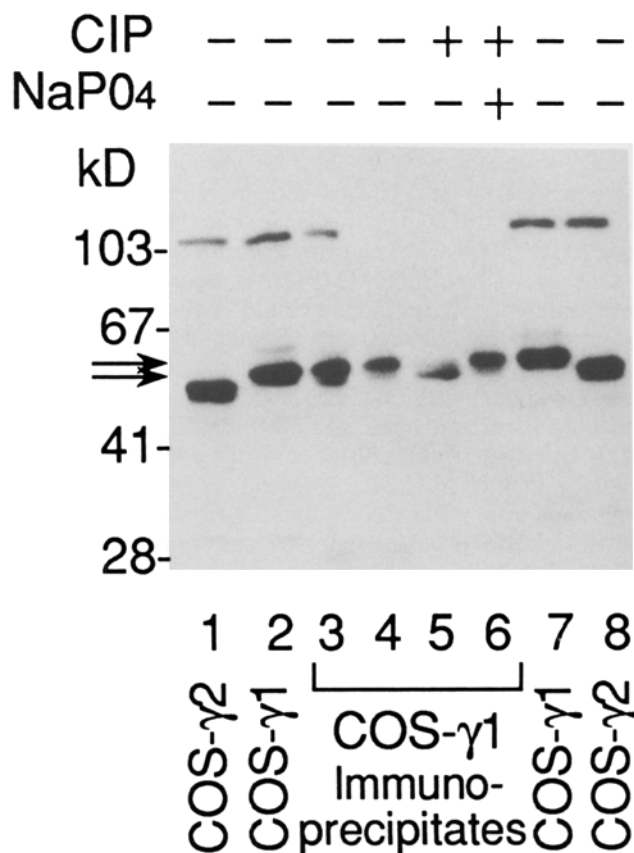

Figure 4. Alkaline phosphatase treatment increases the electrophoretic mobility of mRAR-γ1 protein. Extracts of mRAR-γ1 transfected COS-1 cells were immunoprecipitated using the Ab2γ(mF) mAbs (lanes 3–6) and the antigen–antibody complexes immobilized on Protein A–Sepharose beads were incubated with (lanes 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-γ1 protein was identified by incubation of the filter with RPγ (mF) and [^{125}I]Protein A. In parallel, extracts of COS-1 cells transfected with mRAR-γ1 (lanes 2 and 7) or mRAR-γ2 (lanes 1 and 8) expression vectors were run without prior immunoprecipitation.

and one rabbit polyclonal antiserum directed against the mouse F region (RPγ(mF)), were obtained and were specific for the predominant RAR-γ1 isoform. All these antibodies immunoprecipitate and recognize specifically by immunoblotting a 51-kD protein in nuclear extracts of RAR-γ1-transfected COS-1 cells. This apparent molecular mass is as predicted from the RAR-γ1 cDNA sequence (Krust et al., 1989). This 51-kD protein has a nuclear localization and is absent from cytosolic extracts as confirmed by immunostaining of RAR-γ1 transfected cells. Additionally, in DNA binding assays, the antibodies specifically retard the migration of the complex obtained between extracts of transfected COS-1 cells and the RA response element of the RAR-β2 promoter (RARE-β). These results indicate that our antipeptide antibodies specifically recognize the corresponding epitope of RAR-γ1 protein produced in cells transfected with expression vectors containing the RAR-γ1 cDNAs. The antibodies
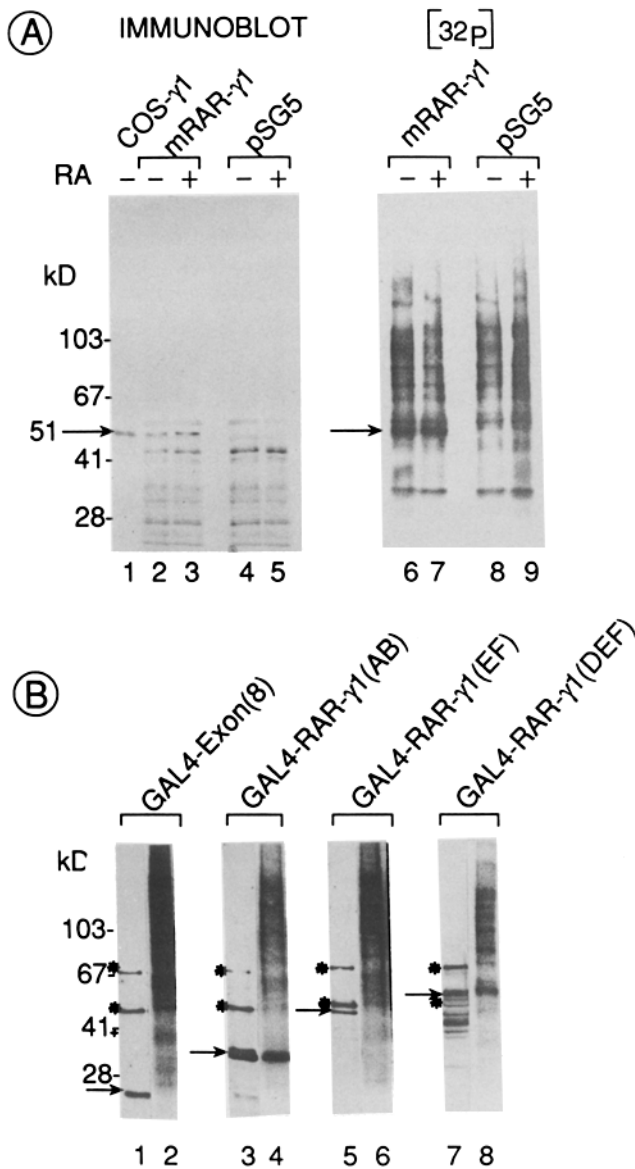
Figure 5. Phosphorylation of mRAR-γ1. (A) COS-1 cells trans-
fected with either pSG5 (lanes 4, 5, 8, and 9) or mRAR-γ1 expres-
sion vector (lanes 2, 3, 6, and 7) were labeled with [³²P] in the
presence or absence of retinoic acid (see Materials and Methods)
and were analyzed by immunoprecipitation with Ab2γ(mF). After
electrophoresis and electrotransfer to NC filters the immunoprecip-
itated phosphorylated proteins were visualized by autoradiography
(lanes 6–9) and identified by incubation of the same NC filter with
RPγ(mF) followed by alkaline phosphatase-labeled antirabbit anti-
bodies (lanes 2–5). Extracts (10 μg protein) of mRAR-γ1 trans-
fected COS-1 cells were run and electroblotted in parallel as a con-
trol (lane 1). (B) COS-1 cells were transfected with the following
chimeric expression vectors: GAL4-Exon(8) (lanes 1 and 2),
GAL4-RAR-γ1(A/B) (lanes 3 and 4), GAL4-RAR-γ1(EF) (lanes 5
and 6), GAL4-RAR-γ1(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. Aster-
isks indicate contaminating immunoglobulins.

directed against the D2 (Ab5γ(D2)) and F(Ab2γ(mF),
Ab4γ(hF) and RPγ(mF)) regions recognized also the RAR-
γ2 isoform, whereas Ab1γ1(A1) did not, in agreement with
the presence of a different A region (A2 in the RAR-γ2
isoform).

The three mAbs Ab1γ1(A1), Ab2γ(mF), and Ab5γ(D2)
recognized either human or mouse RAR-γ proteins. How-
ever, the polyclonal rabbit antibodies RPγ(mF) even though
it was raised against the same peptide SP14 (mouse F region)
that yielded the monoclonal Ab2γ(mF), recognized spe-
cifically the mouse RAR-γ isoforms and not their human
counterparts. Conversely, Ab4γ(hF) was specific for human
RAR-γ proteins, although the sequence of the peptide SP25
used as antigen (human F region) contains amino acid
residues overlapping the sequence of the mouse counterpart
(SP14).

These antibodies allowed the detection of endogenous
RAR-γ isoforms in mouse embryos and F9 embryonal carci-
noma cells nuclear extracts. In both cases mRAR-γ proteins
were not detectable by immunoblotting, but could be immu-
noprecipitated from large amounts (1 mg protein) of nuclear
extracts. Thus, the endogenous mRAR-γ isoforms appear to
be present in low amounts in mouse embryos and F9 cells.
A species corresponding to the mRAR-γ1 isoform (51 kD)
was specifically immunoprecipitated from mouse embryos
nuclear extracts with monoclonal antibodies directed against
either the A1, D2, or F regions. In F9 cells nuclear extracts,
one RAR-γ species with a molecular weight corresponding
to that of the RAR-γ2 isoform (48 kD) was immunoprecipi-
tated as well as a second species with a lower molecular
weight (42 kD). As previously reported for mRAR-γ tran-
scripts (Kastner et al., 1990) the intensity of these signals
was not affected by RA treatment of F9 cells. Since these spe-
cies were specifically immunoprecipitated with Ab2γ(mF)
and Ab5γ(D2), but not with Ab1γ1(A1), they may cor-
respond to the isoform mRAR-γ2 and to a proteolytic prod-
uct of this isoform. Alternatively, the lower species may cor-
respond to the isoforms mRAR-γ5 and/or mRAR-γ6, even
though the corresponding mRNAs appear to be present in
very low amounts in F9 cells (Kastner et al., 1990). Further
studies with specific antibodies directed against the mRAR-
γ2 isoform are required to investigate these possibilities.

The present antibodies allowed us to demonstrate that
RAR-γ1 is modified posttranslationally. mRAR-γ1 appears
to be a phosphoprotein as are steroid hormone receptors (for
review see Aurichhio, 1989). In vitro phosphatase treatment
converted mRAR-γ1 to a faster electrophoretic form as pre-
viously described for the glucocorticoid and progesterone
receptors (Hoeck et al., 1989; Denner et al., 1990a), or for
other transcription factors such as Gal4 (Mylin et al., 1989),
the heat shock transcription factor (Sorger and Pelham,
1988), the adenovirus E1A protein (Dumont et al., 1989;
Smith et al., 1989), the octamer transcription factor (Tanaka
and Herr, 1990), the cAMP-responsive transcription factor
CREB (Gonzalez et al., 1991) and Fos (Ofir et al., 1990).
Furthermore mRAR-γ1 could be labeled in vivo with [³²P],
both in the absence and presence of RA. With the help of chi-
meric constructions in which the Gal4(1-147) DNA binding
domain was fused with the A/B, EF, or DEF regions of
mRAR-γ1, we found that the A/B and most probably the D
regions of RAR-γ1 contain phosphorylation sites. However,
since in the latter case the protein encoded by the Gal4-RAR-

Table II. Protein Kinase Phosphorylation Site Motifs

Protein kinases	Possible phosphorylation recognition motifs
	In the A/B domain
cAMP-dependent kinase	34R G S*
Casein kinase I	40E MLS*
	57E MAS*
	64E TQS*
Casein kinase II	18S*GYP
	67S*T SS
	69S* S EE
Proline-dependent kinase	36S* P
	43S* P
	77S* P
	79S* P
	In the D domain
Casein kinase I	173E EGS*
Casein kinase II	176S* PDS
Glycogen synthase kinase 3	179S*YELS
Proline-dependent kinase	176S* P
	183S* P

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-γ1 (see Kemp and Pearson, 1990) is represented. The phosphate acceptor serines 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.

γ1(DEF) expression vector and not that encoded by Gal4-RAR-γ(EF) 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-γ1 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 (RXSX), proline directed kinase (XSPX), casein kinases I (EXXS) and II (SXXS, SXXE, SXXP), and glycogen synthase kinase 3 (XSXXXSX) (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-γ1 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-γ 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-γ, 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-γ 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-γ 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-γ is obviously required to investigate this and other possibilities.

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