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 Al
(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 RAR-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 Al 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 pleio-
tropic effects of RA are likely to be mediated by specific nu-
clear RA receptors (RARs) which have been discovered in
mouse and human (designated RAR-α, -β, and -γ) (Petko-
vich 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 tran-
scriptional enhancer factors (for reviews see Evans,
1988; Green and Chambon, 1988; Beato, 1989 and refer-
cences 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 mul-
tiple cDNA isoforms for each RAR (Krust et al., 1989; Gi-
guère 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 par-
ticular, the localization of RAR-γ transcripts during embroy-
genesis 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 character-
ization 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

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.

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migrate 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; Zelett 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 (I-147) and the RAR-γ A/B region are IGPRPA. 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(EDF) and (DEF) chimera also contain the amino acids IGPRPA in the linker region. The mRAR-γ 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 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. COS-1 cellswere grown in 9-cm-dia Petridishes, inDMEM, containing 5% FCS, 500 U penicillin, 400 μg gentamycin, and 100 μg streptomycin. Cells were transfected by using the calcium phosphate technique as previously described (Brand et al., 1988). Transfections included either the mouse RAR-γ, 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-γ), 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) were synthesized in solid phase using Fmoc chemistry (model 431A peptides synthesizer, Applied Biosystems, Inc., Foster City, CA) and 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). Cell suspensions 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, Bergancie, France). For ascite fluid production, 2 x 106 cells were injected in pristane-primed Balb/c mice. Class and subclass determination was performed using an Isotyping Kit (Amersham International, Amersham, 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, scraped 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 microcentrators (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 microcentrators (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 at 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), electroblotted 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).

Gal 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-βimin) 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 (2x10^4 cpm) double-stranded [32P]5'-end-labeled synthetic RARE oligodeoxynucleotide and, when requested, 1 μg 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 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 1:1 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 immunoreactive material in 1 ml PBS for 1 h at 4°C. Further incubation for another hour 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. Antibody–antigen 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 MgCl2, 0.1 mM ZnCl2, PIC, and 20 μl of calf intestinal alkaline phosphatase (Boehringer, Mannheim, Germany). Sodium phosphate 10 mM was included as indicator. 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 mCi/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 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 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). The main differences between RAR-γ and RAR-α and β isoforms are located in the NH2-terminal A region, central D2 region, and COOH-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-γ (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.

| 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             |
| RAR-γ1 A1       | B               | C               | D1             | D2             | D3             | E               |
|                |                 |                 |                 |                 |                 |                 |
| Peptide SP15    | Ab1γ1 (A1)     | mouse FEHLSFPSFRGLG | human 39--------50 |
| Peptide SP81    | (D2 region) Ab5γ (D2) |
| mouse           | KEEGPSDAYSETS   |
| human           | 172--------183   |
| Peptide SP14    | (mouse F region) Ab2γ (mF), RPy (mF) |
| mouse           | SSSDEAPGGGKRQG   |
| human           | 436              | 451             |
| Peptide SP25    | (human F region) Ab4γ (hF) |
| mouse           | K-------K-------A |
| human           | QPGFHPNASEDEV    |
| 2               | 51              | 79              | 145             | 191             | 411             | 447             |
| RAR-γ2 A2       | B               | C               | D1             | D2             | D3             | E               |
|                |                 |                 |                 |                 |                 |                 |
| Peptide SP15    | monkey FEHSFPSFRGLG |
| Peptide SP81    | (D2 region) Ab5γ (D2) |
| mouse           | KEEGPSDYSYELS   |
| human           | 39--------50     |

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 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.

**Retinoic Acid Receptor γ: Antibodies and Phosphorylation**

(i) Immunoblotting. The monoclonal antibodies as well as the rabbit polyclonal antiserum were tested for their ability to...
revealed 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 Ab1γ(Al), 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 (Mr = 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 Ab1γ(Al) (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 Ab1γ(Al), 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 Ab1γ(Al), 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 Ab1γ(Al) 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 (Ab1γ(Al), 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 Ab1γ(Al) (Fig. 2 B, lane 17), whereas signals were observed when using the same extracts and either Ab5γ(D2)

Figure 1. Immunocytochemistry of COS-1 cells transfected with mRAR-γ1 using either polyclonal or monoclonal anti-RAR-γ1 antibodies. (A) RPγ(mF); (D) Ab1γ(Al); (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 nonreactive serum (NRS, C) or nonreactive ascite fluid (NRA, G). Immunofluorescence was also performed with Ab1γ(Al) 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.
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 4, 7, 10, and 13) or from COS-1 cells transfected with mRAR-γ1 (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 Abγ1(Al) (lanes 1-3, 16, and 17), Abγ5(Al) (lanes 4-6, 18, and 19), Abβ2γ (mF) (lanes 7-9, 20, and 21), Abγ4γ(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; Abγ1(Al), lanes 5, 10, and 17; Abγ5(Al) (lanes 6, 11, and 18; Abβ2γ(mF), lanes 7, 12, and 19; non-reactive 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 121-Protein A. As positive controls, extracts (10 μg protein) of mRAR-γ1 (COS-1) or mRAR-γ2 (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: Abγ1(Al), lane 3; Abβ2γ(mF), lanes 4, 10 and 16; Abγ5(Al), lane 5; Ab9α(hF), lanes 6 and 11; Abβ2(Al): lanes 7, 12 and 17; Non Reactive Ascite (NRA), lane 13. The monoclonal antibodies Ab9α(hF) and Abβ2(Al) 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: RPy(mF), lanes 1-3, 16-20; Ab1γ(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-γ from extracts of hRAR-γ-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 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 32P-labeled oligodeoxyribonucleotide (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 Ab1γ(Al) 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 Ab1γ(Al) (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 lesser extent with Ab5γ(D2), whereas no shift was observed with Ab1γ(Al) (data not shown). In contrast, mAbs specifically directed against either mRAR-α1 [Ab9α(hF)] or mRAR-α2 [Ab7β(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 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-α 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 Ab1γ(Al), Ab5γ(D2), and Ab2γ(mF) were used (Fig. 3 A, lanes 7–15 and data not shown). However, with the RPγ(mF) antisera 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 synthetized 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 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 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 modifications. Protein phosphorylation often alters mobility of 14.5-d mouse embryos were immunoprecipitated with the mAbs Ab1γ(Al) (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-γ1 proteins were immunoprobed by incubation of the filters with RPγ(mF) and [125I]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 Ab1γ(Al) (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 J 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.
The production, characterization, and use of antipeptide antibodies that are directed against RAR-γ isoforms. Four monoclonal antibodies directed against the Al region (Ab1γ(Al)), the D2 region (Ab5γ(D2)), and the F region (Ab2γ(mF) and Ab4γ(hF)) 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 [3P]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 [3P] 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(DEF) 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 [3P] 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. 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 [125I]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.
directed against the D2 (Ab5γ(D2)) and F(AB2γ(mF), Ab4γ(hF) and RPy(mF)) regions recognized also the RAR-γ2 isoform, whereas Ab1γl(Al) did not, in agreement with the presence of a different A region (A2 in the RAR-γ2 isoform).

The three mAbs Ab1γl(Al), Ab2γ(mF), and Ab5γ(D2) recognized either human or mouse RAR-γ proteins. However, the polyclonal rabbit antibodies RPy(mF) even though it was raised against the same peptide SP14 (mouse F region) that yielded the monoclonal Ab2γ(mF), recognized specifically 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 carcinoma cells nuclear extracts. In both cases mRAR-γ proteins were not detectable by immunoblotting, but could be immunoprecipitated 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 Al, 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 immunoprecipitated as well as a second species with a lower molecular weight (42 kD). As previously reported for mRAR-γ transcripts (Kastner et al., 1990) the intensity of these signals was not affected by RA treatment of F9 cells. Since these species were specifically immunoprecipitated with Ab2γ(mF) and Ab5γ(D2), but not with Ab1γl(Al), they may correspond to the isoform mRAR-γ2 and to a proteolytic product of this isoform. Alternatively, the lower species may correspond 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-γ is modified posttranslationally. mRAR-γ appears to be a phosphoprotein as are steroid hormone receptors (for review see Aurichchio, 1989). In vitro phosphatase treatment converted mRAR-γ1 to a faster electrophoretic form as previously 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 ElA 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]P, both in the absence and presence of RA. With the help of chimeric 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-
Masking 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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References

Auričcio, F. 1989. Phosphorylation of steroid receptors. J. Steroid Biochem. 32:613-622.

Beato, M. 1989. Gene regulation by steroid hormones. Cell. 56:335-344.

Benbrook, D., E. Lernhardt, and M. Pfahl. 1988. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature (Lond.). 333:669-672.

Bedwell, J. E., E. Ott, J. M. Coall, D. J. C. Pappin, L. I. Smith, and F. Swift. 1991. Identification of phosphorylated sites in the mouse glucocorticoid receptor. J. Biol. Chem. 266:7549-7555.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brand, N., M. Petkovich, A. Krust, P. Chambon, H. de Thé, A. Marchio, P. Tiollais, and A. de Jean. 1988. Identification of a second human retinoic acid receptor. Nature (Lond.). 333:850-853.

Brown, T. A., and H. F. Deluca. 1990. Phosphorylation of the 1,25-Dihydroxy-vitamin D receptor. A primary event in 1,25-dihydroxyvitamin D3 action. J. Biol. Chem. 265:10025-10029.

Dollé, P., L. A., W. T. Schrader, B. W. O'Malley, and N. L. Weigel. 1990. Hormonal regulation and identification of chicken progesterone receptor phosphorylation sites. J. Biol. Chem. 265:16548-16555.

Dollé, P., E. Ruberté, P. Kastner, M. Petkovich, C. M. Stoner, L. J. Gudas, and P. Chambon. 1989. Differential expression of genes encoding the retinoic acid receptor β gene. Nature (Lond.). 343:177-180.

Dollé, P., E. Ruberté, P. Leroy, G. Morris-Kay, and P. Chambon. 1990. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. Development. 110:1133-1151.

Dumont, D. J., M. L. Tremblay, and P. E. Brantont. 1989. Phosphorylation at serine 89 induces a shift in gel mobility but has little effect on the function of adenovirus type 5 E1A proteins. J. Virol. 63:987-991.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. Science (Wash. DC). 240:889-895.

Garner, M. M., and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose regulatory system. Nucleic Acids Res. 9:3047-3060.

Gaub, M. P., Y. Lutz, E. Ruberté, M. Petkovich, N. Brand, and P. Chambon. 1989. Antibodies specific to the retinoic acid receptor human nuclear receptors α and β. Proc. Natl. Acad. Sci. USA. 86:3089-3093.

Giguère, V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. Nature (Lond.). 330:624-629.

Table II. Protein Kinase Phosphorylation Site Motifs

| Protein kinases             | Possible phosphorylation recognition motifs            |
|-----------------------------|--------------------------------------------------------|
| In the A/B domain           |                                                        |
| cAMP-dependent kinase       | 34R GS*                                                 |
| Casein kinase I             | 40E MLS*                                                |
|                             | 57E MAS*                                                |
|                             | 64E TQS*                                                |
| Casein kinase II            | 18S+GYP                                                 |
|                             | 67S+TSS                                                 |
|                             | 69S*SEE                                                 |
| Proline-dependent kinase    | 36S*P                                                   |
|                             | 43S*P                                                   |
|                             | 77S*P                                                   |
|                             | 79S*P                                                   |
| In the D domain             |                                                        |
| Casein kinase I             | 176E 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-γ (1,25-Dihydroxy-vitamin D receptor). A primary event in 1,25-dihydroxyvitamin D3 action. J. Biol. Chem. 265:10025-10029.
Gonzalez, G. A., P. Menzel, J. Leonard, W. H. Fischer, and M. R. Montminy. 1989. The c-erbA-encoded thyroid hormone receptor is phosphorylated in its amino terminal domain by casein kinase II. Oncogene. 4:1247–1254.

Goldberg, Y., C. Glineur, J. Cesquiere, A. Ricouart, J. Sap, B. Venström, and J. Ghyssael. 1988. Activation of protein kinase C or c-AMP-dependent protein kinase increases phosphorylation of the c-erbA-encoded thyroid hormone receptor and of the v-erbA-encoded protein. EMBO (Eur. Mol. Biol. Organ.) J. 7:2425–2433.

Goncalves, R., A. Menzel, J. Leonard, W. H. Fischer, and M. R. Montminy. 1991. Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. Mol. Cell. Biol. 11:1306–1312.

Green, S., and P. Chambon. 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. 4:309–314.

Hoeck, W., and B. Groner. 1990. Hormone dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino terminal transactivation domain. J. Biol. Chem. 265:5403–5408.

Hoeck, W., S. Rusconi, and B. Groner. 1989. Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. J. Biol. Chem. 264:14396–14402.

Kastner, P., A. Krust, C. Mendelsohn, J. M. Garnier, A. Zelent, P. Leroy, A. Staib, and P. Chambon. 1990. Murine isoforms of retinoic acid receptor α with specific patterns of expression. Proc. Natl. Acad. Sci. USA. 87:2700–2704.

Kemp, B. E., and R. B. Pearson. 1990. Protein kinase recognition sequence motifs. Trends Biol. Sci. 15:342–346.

Krust, A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon. 1989. A third human retinoic acid receptor hRAR-γ. Proc. Natl. Acad. Sci. USA. 86:5310–5314.

Leroy, P., A. Krust, A. Zelent, C. Mendelsohn, J. M. Garnier, P. Kastner, A. Dietrich, and P. Chambon. 1991. Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and differential induction by retinoic acid. EMBO (Eur. Mol. Biol. Organ.) J. 10:59–69.

Lutz, Y., M. Jacob, and J. P. Fuchs. 1988. The distribution of two hnRNP-associated proteins defined by a monoclonal antibody is altered in heat-shocked HeLa cells. Exp. Cell. Res. 175:109–124.

Maden, M. 1982. Vitamin A and pattern formation in the regenerating limb. Nature (Lond.). 295:672–675.

Mytil, L. M., J. P. Bhat, and J. E. Hopper. 1989. Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. Genes & Dev. 3:1157–1165.

Nicholson, R. C., S. Mader, S. Nagpal, M. Leid, C. Rochette-Egly, and P. Chambon. 1990. Negative regulation of the rat streptomycin gene promoter by retinoic acid is mediated by an API binding site. EMBO (Eur. Mol. Biol. Organ.) J. 9:4443–4454.

Ofir, R., V. J. Dwarki, D. Rashid, and J. M. Verma. 1990. Phosphorylation of the C terminus of Fos protein is required for transcriptional transactivation of the c-fos promoter. Nature (Lond.). 348:80–82.

Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (Lond.). 330:444–450.

Riba, H. P., D. A. Jans, H. Pan, and R. Peters. 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. EMBO (Eur. Mol. Biol. Organ.) J. 10:633–639.

Rochette-Egly, C., C. Fromental, and P. Chambon. 1990. General repression of enhancer activity by the adenovirus-2 EIA proteins. Genes & Dev. 4:137–150.

Rutber, E., P. Dolfé, A. Krust, A. Zelent, G. Morriss-Kay, and P. Chambon. 1990. Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. Development. 108:213–222.

Rutber, E., P. Dolfé, P. Chambon, and G. Morriss-Kay. 1991. Retinoic acid receptors and cellular retinoid binding proteins II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development. 111:45–60.

Smith, C. L., C. Debouck, M. Rosenberg, and J. S. Culp. 1989. Phosphorylation of serine residue 89 of human adenosine E1A proteins is responsible for their characteristic electrophoretic mobility shifts, and its mutation affects biological function. J. Virol. 63:1569–1577.

Sorger, P. K., and H. R. B. Pelham. 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell. 54:855–864.

St. Groth, F. S., and D. Scheiddegger. 1980. Production of monoclonal antibodies: strategy and tools. J. Immunol. Methods. 35:1–21.

Sucov, H. M., K. K. Murakami, and R. M. Evans. 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. Proc. Natl. Acad. Sci. USA. 87:5392–5396.

Tanaka, M., and W. Herr. 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell. 60:375–386.

Thaller, C., and G. Eichele. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature (Lond.). 327:625–628.

Vasios, G., S. Mader, J. D. Gold, M. Leid, Y. Lutz, M.-P. Gaub, P. Chambon, and L. Grades. 1991. The late retinoic acid induction of laminin B1 gene transcription involves RAR binding to the responsive elements. EMBO (Eur. Mol. Biol. Organ.) J. 10:1149–1158.

Webster, N. J. G., S. Green, D. Tasset, M. Ponglikitmongkol, and P. Chambon. 1989. The transcriptional activation function located in the hormone-binding domain of the human oestrogen receptor is not encoded in a single exon. EMBO (Eur. Mol. Biol. Organ.) J. 8:1441–1446.

Zelent, A., A. Krust, M. Petkovich, P. Kastner, and P. Chambon. 1989. Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. Nature (Lond.). 339:714–717.

Zelent, A., C. Mendelsohn, P. Kastner, A. Krust, J.-M. Garnier, F. Ruffenach, P. Leroy, and P. Chambon. 1991. Differentially expressed isoforms of the mouse retinoic acid receptor β are generated by usage of two promoters and alternative splicing. EMBO (Eur. Mol. Biol. Organ.) J. 10:71–81.