Pergamon

J Steroid Biochem Molec Biol Vol 48, No 1, pp 61–68, 1994

0960-0760/94 $6.00 + 0.00

Immunolocalization of Retinoic Acid Receptors in Rat, Mouse and Human Ovary and Uterus

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We raised an antibody against a synthetic peptide corresponding to amino acids 155–174 of human retinoic acid receptor α (RAR-α). The sequence is highly homologous in all RARs and their isoforms. When mouse and human RARs (α, β and γ) expressed in Cos cell were analysed with immunoblot, all receptors gave a specific 51 K signal. Mouse RAR-γ gave an additional signal corresponding to 58 K. In human teratocarcinoma cells (F9) both 51 and 58 K molecule sizes were detected. The RAR expression in F9 cells was slightly down-regulated in charcoal-stripped culture medium and returned to normal level after retinoic acid treatment. The 51 K protein was found in all ovarian and uterine samples, but the quantity of the 58 K protein varied in different species and organs, being highest in the mouse uterus and the rat and human ovary. Using immunohistochemistry the RARs were found in the nuclear compartment. In the rat uterus, positive immunoreaction was found mainly in the nuclei of epithelial, uterine glandular and stromal cells. In the rat ovary, positive reaction was found in the nuclei of germinal epithelial, follicular and stromal cells.

J. Steroid Biochem. Molec. Biol., Vol. 48, No. 1, pp. 61–68, 1994

INTRODUCTION

Retinoic acid (RA) has a variety of effects on the growth and differentiation of normal and malignant cells. RA induces the vertebrate limb morphogenesis and affects foetal development [1–3]. RA is also an important regulator of terminal differentiation at various stages of keratinization in epidermal cells [4, 5], and induces differentiation of some malignant tumour cells, such as human leukemic HL-60, murine S91-C2 melanoma cells and F9 human teratocarcinoma cells [2, 6, 7].

Retinoic acid receptors (RARs) belong to the nuclear steroid and thyroid hormone receptor superfamily, which act as ligand-dependent transcription factors in target genes by binding to specific regulatory sequences known as retinoic acid response elements (RAREs). There are three different RARs (RAR-α, β and γ), and several isoforms which are generated by differential splicing of exons encoding the A domain [8, 9]. During embryogenesis, three different RAR mRNAs were expressed in the developmental stage and in an organ-specific manner [10–12]. In the adult animal tissues, expression of RAR-α mRNA is universal and RAR-β is more restricted in certain organs, while RAR-γ seems to be predominantly expressed in skin and lung [8, 9, 13–15]. To date there are, however, no data on the distribution of the RAR protein in different organs.

The regulation of RAR-α, β and γ by their ligands varies in different cells and tissues. F9 and P19 teratocarcinoma cells have been widely used as a model for studying cell differentiation and retinoid action [7]. In F9 cells, the expression of RAR-β mRNA was increased 20-fold after RA treatment, but RAR-α and γ are expressed constantly while in P19 cells RAR-α is up-regulated by RA treatment [9, 17].

Although RARs have been found to be crucial for spermatogenesis and maintenance of pregnancy [15, 16, 18], their specific actions in the reproductive organs are still not known. In this study, we report the generation and characterization of a rabbit polyclonal antibody against a synthetic peptide which is similar in all three RARs. By means of this antibody, we studied the expression of RAR proteins in F9 teratocarcinoma cells in human, mouse and rat ovary and uterus by both immunoblotting and immunohistochemistry. The regulation of RARs by RA and retinol was also studied.

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Received 1 June 1993; accepted 14 Sep. 1993.

MATERIALS AND METHODS

Antibody

The polyclonal antibody used in this study was RAR82, which was made against a synthetic peptide P17 (human RAR-α amino acid 155–174: lys-glu-ser-val-arg-asn-asp-arg-asn-lys-lys-lys-lys-glu-val-pro-lys-pro-glu-cys). This sequence is derived from the N-terminal (D region) of human RAR-α [9]. It was chosen according to its hydrophilic and antigenic index. The only difference in this sequence between human and mouse RAR-α is the single amino acid change from $Valine_{169}$ (Val/human) to $Alanine_{169}$ (Ala/mouse). A second criterion for choosing this sequence was its high homology with both human and mouse RAR-β and γ. A comparison of the amino acid sequence of human RAR-α155–174 with corresponding sequences of human and mouse RAR-β and γ is given in Fig. 1. Rabbit immunization and antisera preparation were done as follows: California rabbits were initially injected with 100 μg of peptide coupled with thyroglobulin in 0.5 ml of Freund's complete adjuvant at three sites under the nail skin. The booster injections were repeated five times with Freund's incomplete adjuvant at 1-month intervals. Rabbit blood was collected from the ear vein, 10 days after boosts. Serum was collected from second and subsequent bleedings. Serum was separated by low speed centrifugation (2000 *g* for 15 min). Immunoglobulin fraction G (IgG) was precipitated with 40% ammonium sulphate. The precipitate was dissolved in distilled water and dialyzed in phosphate buffered saline (PBS) for about 36 h. The concentration of IgG was adjusted to 1 mg/ml in stock solution with 0.05% NaN_3. The titres of antibody were measured using ELISA. Western blot was used to test the ability of this antibody to recognize the corresponding peptide.

Cell culture and RARs cDNA transfection

Cos cells were cultured in Dulbecco's modified Eagle's minimum medium (DMEM) culture medium (GIBCO, England). Whole length human and mouse RAR cDNAs subcloned into the eukaryotic expression vector pSG5 [19], were transfected by a standard calcium-phosphate technique [20]. Human RAR-α, a generous gift from Jian-Yang Chen (manuscript in preparation), was expressed in *E. coli* by using a prokaryotic expression vector pET3A [21]. cDNAs of both human and mouse RAR-α, β and γ were kindly provided by Professor P. Chambon of the Institute de Chimie Biologique (Faculté de Médecine, Strasbourg, France).

F9 human teratocarcinoma cells were cultured in DMEM containing 10% foetal calf serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin for 3 days. Cells were cultured in charcoal-stripped medium for at least 24 h, whereafter 1 μM all *trans* RA or retinol (Sigma Chemical Co., St Louis, MO, U.S.A.) was added to culture medium 3 and 24 h before cells were collected.

Mouse, rat and human tissues

Human ovary and uterus were from the Ostetrics and Gynaecology Department, the University Hospital of Tampere, with the permission of the local Ethical Committee. Adult female rats (Wistar) and mice (Normal Medical Research Institute, NMRI) were used. Ovary and uterus were taken immediately after the rats and mice were killed by decapitation. All human, mouse and rat tissues were frozen in liquid nitrogen and stored at −70°C for immunoblotting and cryosectioning.

Cell and tissue extracts

Transfected Cos cells were harvested and taken into extraction buffer containing 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 0.4 M NaCl, and 10% glycerol, and then broken by freeze–thawing in liquid nitrogen and on ice. The homogenates were centrifuged at 16,000 *g* for 15 min. The supernatant was taken as cytosol. F9 cells were homogenized in 4 vol of homogenization buffer containing 10 mM Tris–HCl pH 8, 20 mM sodium molybdate, 1.5 mM EDTA, 1 mM PMSF. After centrifugation at 16,000 *g* for 15 min, the supernatant was taken as cytosol. The pellets were further incubated in homogenization buffer containing 0.6 M KCl for 1 h, and then centrifuged at 100,000 *g* for 1 h. The supernatant was taken as nuclear extract. Both homogenization buffers contained protease inhibitor cocktail (leupeptin 1.25 mM, bacitracin 10 mg/ml, aprolinin 5 mg/ml and pepstatin 1 mM).

The whole tissue extracts from human, rat and mouse tissues were prepared similarly as extracts from F9 cells, except that the homogenization buffer contained 0.6 M KCl. The homogenates were centrifuged at 100,000 *g* for 1 h, and the supernatant was taken as tissue extract. All procedures were carried out at 4°C.

Immunoblot

The supernatants from cells and tissues were boiled in 4 vol of sample buffer (50 mM Tris, 1% SDS, 10%

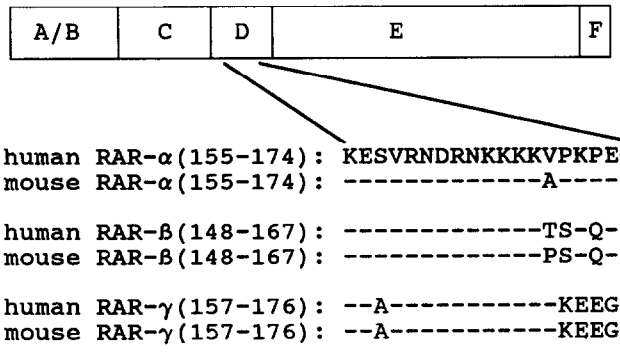

Fig. 1. Comparison of amino acid sequence of peptide P17 (human RAR-α155–174) with corresponding amino acid sequences of human and mouse RAR-α, β and γ.

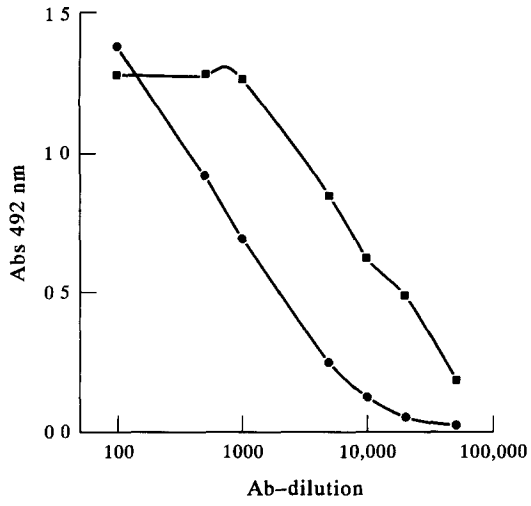

Fig. 2. Dilution curve of the rabbit antiserum and IgG against a synthetic peptide P17 derived from human RAR-α. Different dilutions of antiserum and IgG (RAR82) were tested against peptide P17 coated on a microtitre plate with 0.1% glutaraldehyde. ■—■, Antiserum dilutions (1:100–1:50,000). ●—●, IgG dilutions (10 μg/ml–20 ng/ml).

glycerol, 5.5% dithiothreitol) for 4 min. Protein samples were resolved in 12% polyacrylamide slab gels containing 0.1% SDS according to the method of Laemmli [22]. Electrophoresis was performed at room temperature at 200 V for 45 min, using a Mini-Protean II Dual Slab Cell (Bio-Rad Labs, CA, U.S.A.). The protein was transferred from the SDS–polyacrylamide gel to nitrocellulose sheets with an electrophoretic transfer apparatus (Bio-Rad) at 150 mA for 1 h in transfer buffer [25 mM Tris, 192 mM glycin, 20% (v/v) methanol]. Primary antibody (RAR82) were incubated at 4°C overnight at a concentration of 5 μg/ml in diluting buffer (1% nonfat milk powder in Tris buffer containing 0.05% Tween-20). After extensive washings in Tris buffer containing 0.05% Tween-20, alkaline phosphatase-conjugated swine antirabbit secondary antibody (1:500, Orion Diagnostica, Espoo, Finland) or peroxidase-conjugated goat antirabbit secondary antibody (1:10,000, Cappel, Organon Teknika Corp., PA, U.S.A.) was added for 1 h at 37°C or at room temperature. Visualization was carried out using Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP, Sigma) or enhanced chemiluminescence (ECL, Amersham International, Amersham, England) according to the manufacturer's instructions. Molecular weight standards were phosphorylase *b* M_r 97 K, bovine serum albumin (BSA) M_r 66 K, Ovalbumin M_r 45 K, carbonic anhydrase M_r 31 K, soybean trypsin inhibitor M_r 21 K and lysozyme M_r 14 K (Bio-Rad).

Immunohistochemistry

Frozen sections (7 μm) were cut on a cryostat (Microm, Heidelberg, Germany) at −20°C and thawed on glass coated with poly-D-lysine. The sections were fixed in Zamboni's fixative (2% paraformaldehyde in 15% saturated aqueous picric acid) for 10 min at room temperature. After rinsing twice in PBS, sections were blocked with 10% normal goat serum (NGS) for 15 min at room temperature. They were then incubated with polyclonal antibody (RAR82) at a concentration of 2 μg/ml with PBS containing 1% NGS overnight at 4°C, and after washings with PBS treated with biotinylated goat antirabbit antibody (1:1000, Vector Lab, Burlingame, CA, U.S.A.) for 30 min at room temperature. Visualization was carried out using the

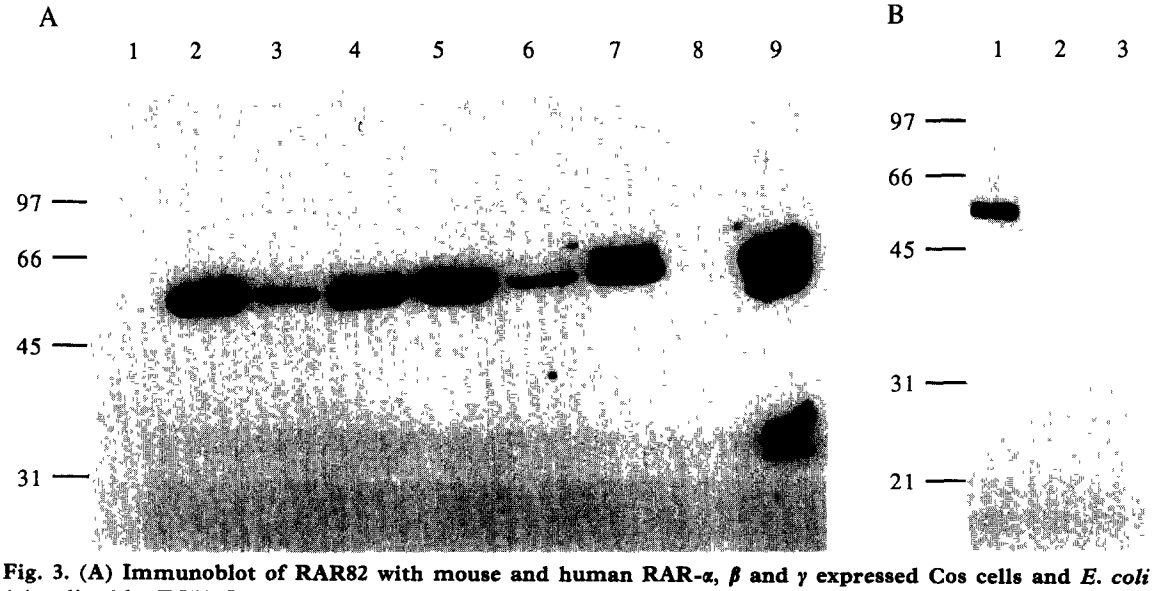

Fig. 3. (A) Immunoblot of RAR82 with mouse and human RAR-α, β and γ expressed Cos cells and *E. coli* (visualized by ECL). In transfected Cos cells, a single band of 51 kDa was seen in human and mouse RAR-α, β and γ. An additional band of 58 kDa was found in mouse RAR-γ. In transfected *E. coli*, 51 and 48 kDa bands were seen in human RAR-α. Lane 1: nontransfected Cos cells; lanes 2–4: human RAR-α, β and γ; lanes 5–7: mouse RAR-α, β and γ. Lane 8: non-transfected *E. coli*; lane 9: human RAR-α transfected *E. coli*. (B) Immunoblot of human RAR-α cDNA-expressed Cos cells with RAR82 (lane 1); primary antibody was preabsorbed with excess peptide P17 (lane 2); primary antibody was replaced by preimmune rabbit IgG (lane 3).

avidin–biotin peroxidase complex (Vector) for 30 min, and 0.05% diaminobenzine tetrahydrochloride (DAB) as chromogen with 0.01% imidazole and 0.01% H_2O_2 in 0.5 M Tris, pH 7.6 for 5 min. No counterstain was used.

Controls

Controls for both immunoblotting and immunohistochemistry included substitution of the primary antibody with (1) PBS with 1% NGS and (2) purified preimmune rabbit IgG at the same concentration as primary antibody; (3) presaturation of the primary antibody with an excess of the corresponding peptide; and (4) presaturation of the primary antibody with the cytosol of human RAR-α expressed Cos cells.

RESULTS

Generation of the antibody to RARs

The beginning of the domain D is highly conserved in all RARs and their isoforms. In order to generate an antibody which recognizes all RARs, we selected an immunogenic amino acid sequence which is located in the D1 region of human RAR-α155-174. The human and mouse sequence in this region differs by only one amino acid. There is a 3 to 6 amino acid difference in this sequence between RAR-α, β and γ (Fig. 1). This peptide conjugated to thyroglobulin was successfully used to generate a rabbit polyclonal antibody (RAR82). It recognized specifically the peptide on Western blot (data not shown). When the titre of the IgG fraction and antiserum to free peptide was tested by ELISA, the antibody had a high titre (Fig. 2).

Specific detection of human and mouse RARs proteins in transfected Cos cells and E. coli

RAR expressed in Cos cells and *E. coli* was used to test this antibody (RAR82) in immunoblotting. Figure 3(A) shows that it recognized a single protein of 51 K with human and mouse RAR-α (lanes: 2,5) β (lanes: 3, 6) and human RAR-γ (lanes: 4, 7) expressed in Cos cells (lanes 2–7). In Cos cells transfected with mouse RAR-γ, it recognized a higher M_r signal of about 58 K as well as 51 K. Human RAR-α cDNA expressed *E. coli* showed a doublet with M_r of about 51 and 48 K. In addition, it also showed two smaller M_r bands about 30 K which were probably proteolytic fragments [Fig. 3(A), lane: 9]. All these signals were specific

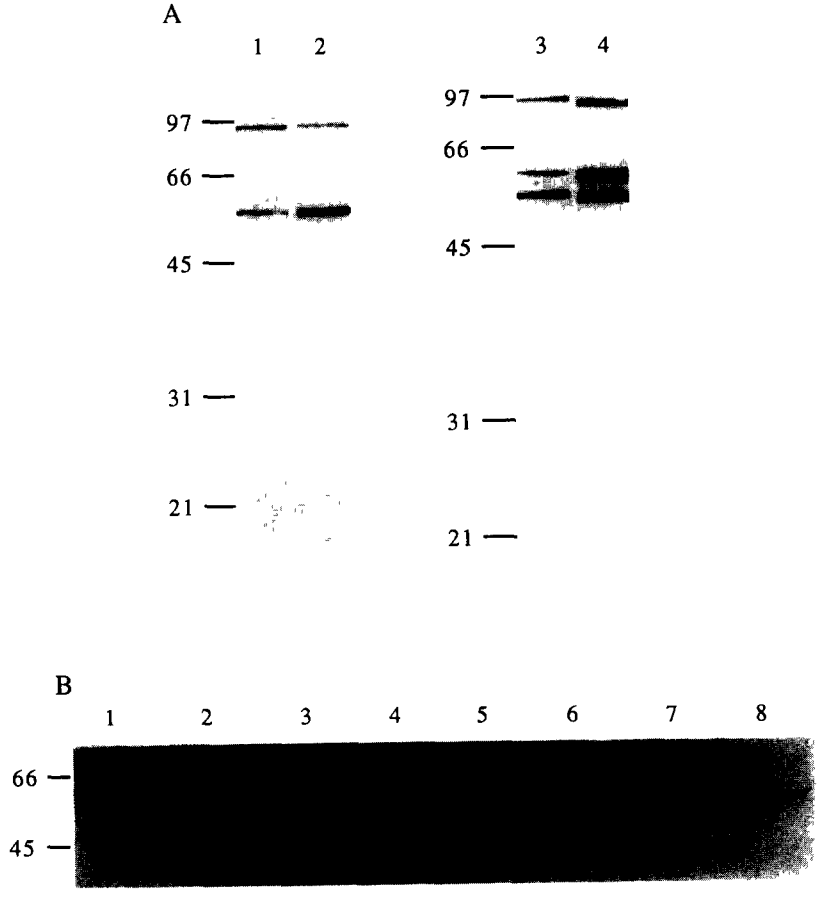

Fig. 4. Immunoblot of RAR82 with F9 human teratocarcinoma cells. (A) M_r of 51 and 58 kDa bands were seen in both cytosol (lanes: 1, 3) and nuclear extracts (lanes: 2, 4). An approx. 95 kDa protein was also seen in F9 cells. The 58 kDa bands were much stronger after precipitation (lanes: 3, 4) than before (visualized by ECL). (B) Regulation of RAR proteins in F9 cells by RA. The RAR protein concentration was slightly decreased after culture in charcoal-stripped medium, and close to normal level after RA treatment. Lanes 1–4: cytosol; lanes 5–8: nuclear extracts; lanes 1, 5: F9 cells in normal medium; lanes 2, 6: steroid-free medium for 24 h; lanes 3, 7: 3 h and 24 h (lanes 4, 8) after RA treatment (visualized by NBT/BCIP).

because only a very weak band (51 K) was found in nontransfected Cos cells and no signal in nontransfected *E. coli* [Fig. 3(A), lanes 1 and 8]. The specificity of RAR82 was also tested on immunoblot with the primary antibody presaturated with an excess of the corresponding peptide (P17) or replaced by preimmune rabbit IgG. No specific signals were seen in these experiments [Fig. 3(B)].

RARs in F9 teratocarcinoma cells

The cytosol and nuclear extracts of F9 cells were studied on immunoblot with RAR82. The two molecular forms (51 and 58 K) were shown in both cytosol and nuclear extracts [Fig. 4(A)]. The antibody also recognized a large protein of about 95 K. The intensity of the 58 K protein was much lower than 51 K. After 50% ammonium sulphate precipitation, the 58 kDa protein became more intense while 51 kDa showed little change [Fig. 4(A), lanes 3 and 4].

The regulation of RAR proteins by its ligands was studied in F9 cells. The 58 K form in both cytosol and nuclear extracts became slightly weaker after culture in charcoal-stripped medium instead of normal medium. Three and 24 h after all *trans* RA (1 μM/ml) was added to culture medium, the intensity of the 58 K signal increased close to normal level [Fig. 4(B)]. The RAR concentration was unaltered when all *trans* retinol (1 μM/ml) was used (data not shown).

RARs in mouse, rat and human ovary and uterus

The expression of RARs in mouse, rat and human ovary and uterus was studied by both immunoblot and immunohistochemistry. In immunoblot, a 51 K protein was found in all mouse, rat and human samples, in ovary as well as uterus (Fig. 5), whereas the 58 K protein was shown mainly in mouse uterus, rat ovary and human ovary (Fig. 5, lanes; 2, 3, 5). We found the 58 K form in mouse ovary, rat uterus and human uterus only if we prolonged exposure of the blots.

In immunohistochemistry, a positive immunoreaction was seen mainly in the nuclei of target cells. In the rat uterus, immunostaining was found in the nuclei of epithelial, stromal and uterine glandular cells [Fig. 6(A and B)]. The endothelial cells in blood vessels also showed nuclear staining [Fig. 6(C)]. Muscle cells also had positive staining in nuclei, but the cytoplasmic staining was also high. It is not known whether this cytoplasmic staining is specific.

In the rat ovary, the strongest immunostaining was found in the nuclei of germinal epithelial cells [Fig. 6(E)]. Positive staining was also shown in the follicular cells [Fig. 6(F)]. In the follicles, the highest concentration of RARs was detected in the granulosa cells, of which most showed positive staining. In the thecal layer only a few cells showed detectable staining. The follicular cells in the primary and secondary follicles had stronger positive reaction than in the mature follicles. Follicular cells in the atretic follicle also showed positive reaction. Granulosa lutein cells in the corpus luteum showed only very weak positive reaction. Immunoreaction was also found in some stromal cells [Fig. 6(G)].

Control stainings were done by replacing the primary antibody with PBS or, preimmune rabbit IgG, or presaturating primary antibody with cytosol of transfected Cos cells [Fig. 6(D)]. No specific staining was found in any of these controls.

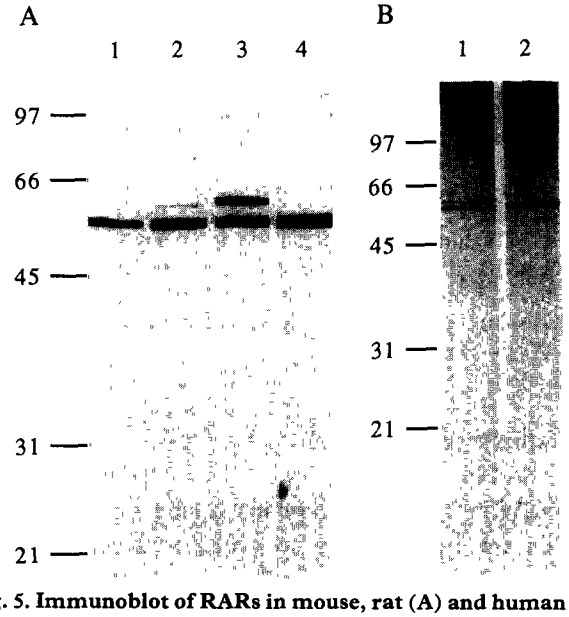

Fig. 5. Immunoblot of RARs in mouse, rat (A) and human (B) ovary and uterus. A M_r of 51 kDa protein band was detected in all samples, but the 58 kDa band was only found in mouse uterus, rat and human ovary. (A) Mouse (lane 1) and rat (lane 3) ovary; mouse (lane 2) and rat (lane 4) uterus (visualized by ECL). (B) Human ovary (lane 1) and uterus (lane 2) (visualized by NBT/BCIP).

DISCUSSION

We raised an antibody (RAR82) against a synthetic peptide deduced from a cDNA sequence of human RAR-α (amino acid 155–174). The sequence is located between the DNA-binding and the RA-binding domain and there is considerable homology between the various classes of RARs in this region [9]. The antibody recognized specifically human RAR-α expressed in *E. coli* and human and mouse RAR-α expressed in Cos cells. It also reacted with similar sensitivity as the RAR-α, but was considerably less sensitive in detecting RAR-β.

In untransfected Cos cells, the antibody detected a faint band of 51 K, apparently an endogenous RAR. This signal was markedly increased after transfecting various RAR cDNA. In addition to the 51 K protein, two other proteins (58 and 95 K) were detected in F9 cells and in mouse, rat and human genital organs. The 51 K signal probably represents the form which is common to all RARs and the 58 K signal one form of the RAR-α [23–28]. High M_r signals comparable to 95 K detected with our antibody have also been detected with other RAR antibodies [25, 28]. The origin of this signal, which markedly exceeds the calculated M_r of RARs (50–51 K), is not known.

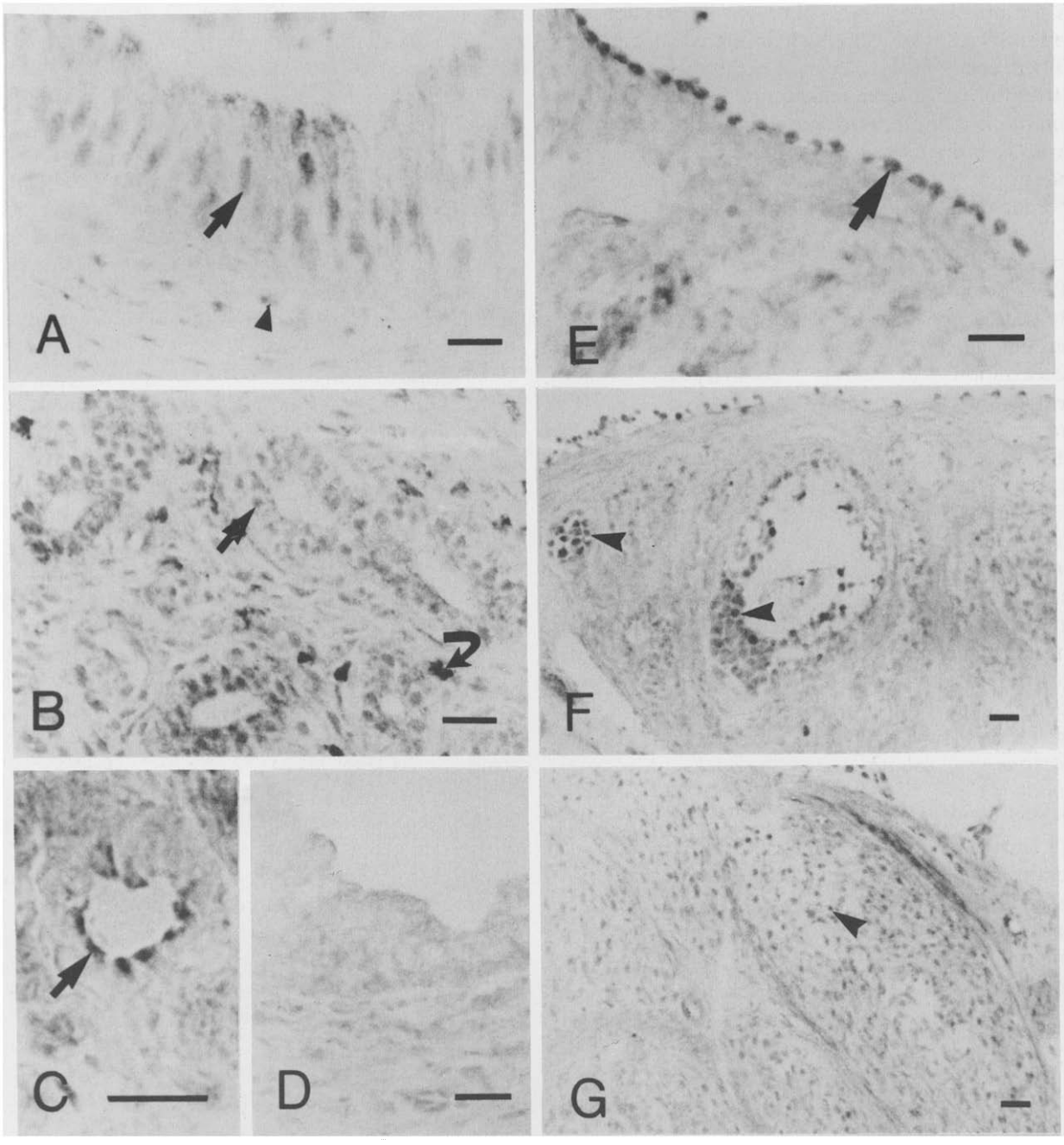

Fig. 6. Immunohistochemistry of RARs in rat uterus (A–C) and ovary (E–G). In the uterus, immunopositive staining was located in the nuclei of (A) epithelial (arrow) and stromal cells (arrowhead). (B) Uterine glandular cells (arrow), curved arrow: endogenous peroxidase. (C) Endothelial cells of blood vessels (arrow). In the ovary, immunopositive reaction was found in the nuclei of (E) germinal cells (arrow), (F) follicular cells (arrowhead), (G) follicular cells in atretic follicle (arrowhead). Control staining (D) was done: primary antibody was presaturated with the cytosol of Cos cells expressing human RAR-α. No specific staining was seen. Bar equals 25 μm.

The quantity of the 58 K form varied greatly from tissue to tissue, being lowest in mouse ovary and rat and human uterus, whereas the 51 and 58 K signals were equally strong in rat and human ovary.

Like the other members of the steroid hormone receptor family, RARs are regulated by their own ligands. RAR-β transcript is up-regulated by RA in F9 cells and in the tissues of vitamin A deficient rats, while RAR-γ is only found to be up-regulated in the rat skin [9, 15, 16]. In the rat gonad, Kim *et al.* [18] reported that mRNA of RAR-α in the vitamin A-deficient testis is rapidly increased after treatment with retinol, whereas Haq *et al.* [15] and Kato *et al.* [16] found that the RAR-α transcript remains unchanged in vitamin A-deficient rat testis after treatment with retinol or RA. Little is known of the regulation of RARs at protein level. Recently, Gaub *et al.* [28] found that RAR-α is weakly up-regulated by RA in F9 cells, and a new protein signal (42 K) appears after RA treatment. In our study, RARs were down-regulated in charcoal-stripped culture medium and weakly up-regulated after

RA treatment in F9 cells. The 58 K form was more affected by charcoal stripping and RA treatment.

There are several *in situ* hybridization studies on the distribution of the RAR transcripts. However, there are no immunohistochemical studies on the distribution of the RAR proteins. The existence of cellular retinoid binding proteins makes it difficult to study the RARs by autoradiography [29]. The apparently low cellular concentration of the RARs hinders their detection by immunohistochemistry [30, 31]. We were, however, able to detect RAR in immunohistochemistry, probably because we have raised an antibody which recognizes all of the RAR forms and thus the epitope concentration is higher.

Retinoids are required for normal reproduction. Vitamin A deficiency leads to foetal resorption in pregnant rats and degeneration of testicular germ cells [32, 33]. The maintenance of the normal reproductive function has been shown to require retinol, whereas RA substitution is not sufficient to restore totally normal reproduction in vitamin A-deficient animals [32]. The mechanism by which retinoids regulate gonads is not known. They are known to increase progesterone synthesis in the granulosa cell *in vitro* [34, 35]. *In vivo* progesterone is mainly synthetized in granulosa lutein and theca lutein cells of the corpus luteum, which is formed from ovarian follicles after ovulation. Progesterone and androgen receptors have been found in granulosa follicular and lutein cells [36–38]. We have detected RARs in ovarian follicles, both in the granulosa and thecal cells. The germinal epithelium also contains high concentration of RARs as well as progesterone and androgen receptors [38, 39]. The function of these cells and their regulation by steroids remains, however, unclarified.

There is no information as to the distribution of RARs in the genital region after the onset of organogenesis nor the modulation of the sex-steroid-induced genital differentiation by retinoids. Retinoids are, however, known to modulate cellular sensitivity to progestins and estrogens. RA treatment is known to decrease the expression of progesterone receptor at the mRNA and protein level in T47D cells [40, 41]. It also has antiestrogen- and antiprogestin-like effects in decreasing the progesterone and estrogen receptor-mediated transcription activation [42]. We have shown that all of the cell types in the uterus contain RARs. The concentration is highest in the glandular epithelial cells and lowest in the stromal cells. The same cells are also known to contain progesterone and estrogen receptors [43]. It is thus possible that retinoids regulate the uterus by modulating the functions of sex steroids as well as directly through their own receptors.

Acknowledgements—The authors would like to thank Anja Rovio and Hilkka Mäkinen for excellent technical assistance and Robert MacGilleon for revising the language We are grateful to Professor Pierre Chambon for his gift of cDNAs of RAR-α, β and γ and RAR-α protein expressed in *E. coli*. This work was supported by grants from the Sigrid Juselius Foundation and the Academy of Finland

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