Development of progestin-specific response in the chicken oviduct

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ABSTRACT. Avidin is a host acute defense protein induced by progestins and by inflammation caused by injurious factors such as microbes, viruses, toxic factors or tissue trauma. In the reproductive tract of egg-laying vertebrates avidin has evolved into a progestin-dependent secretory protein involved in antimicrobial action through its biotin avidity. For *«progestin-dependent avidin»* production, cellular differentiation by estrogen is necessary. In contrast, the expression of *«progestin-independent or inflammation-induced avidin»* does not require differentiation. Many cell types such as macrophages, heterophils and fibroblasts can produce avidin after non-specific cellular injuries. The wide distribution of avidin in avian, reptilian and amphibian species could be explained on the basis of its vital functions such as antimicrobial or antifungal, metabolic and immunomodulatory actions.

The ontogeny of the progestin-dependent avidin synthesis is a complex event involving oviductal differentiation by steroid hormones leading to a a specific gene expression. The first phase in oviductal differentiation by estrogens is characterized by a new chromatin organization and by an infiltration of progesterone receptor (PR)-containing mesenchymal cells into the subepithelial mucosa leading to epithelial cell differentiation *(«mesenchymal and epithelial cell interaction»)*.

The second phase in the differentiation of progestin-induced response is dependent on the presence of PR in the secretory cells. Two kinds of PR expression occur in the oviduct. The first is a *«constitutive PR»* and is found in the epithelial, submucosal and peritoneal cells of the immature chick oviduct without steroid treatment, and the second is an *«inducible PR»* found especially in the mucosal mesenchymal and smooth muscle cells. Avidin production requires PR in the target cells, but not all PR-containing cells can produce avidin. Therefore, in addition to PR, other transcription factors are needed to define the target cell specificity of the response to progestins.

Earlier biochemical studies suggested that cytosolic and/or nuclear unoccupied PR was complexed as an 8 S form with the heat shock protein 90 (hsp90). Our immunohistochemical results, however, indicate that PR *in vivo* is not bound to hsp90, which is located entirely in the cytoplasm, whereas PR is an entirely nuclear protein in both ligand-occupied and unoccupied forms. Therefore, we assume that PR is a monomeric (4S) or homodimeric (5S) (chromatin?) protein associated to DNA. Ligand binding to PR appears to lead to a conformational change, dimer formation, tighter binding to PRE (progesterone responsive element) and to transcription factors, phosphorylation and proteolysis of PR as well as a chromatin change. These events are reflected in the immunohistochemistry as a decrease in immunoreactive PR *(«PR down-regulation»)* during the transcription of the progestin-specific genes. Because no transformation from 8S to 4S form nor a translocation of PR from the cytoplasm into the nucleus occurs, we propose the *«one-step nuclear model»* of progestin action.

A full-length cDNA for egg-white avidin has been cloned. The avidin gene appears to be regulated mainly at the transcriptional level. Molecular cloning of the chromosomal gene indicates that in addition to the progestin-regulated *egg-white avidin gene*, there are at least *five avidin-related genes*. The chicken egg-white avidin gene and its 5'-flanking region provide a model system to study interaction of PR, PRE and transcription factors in the formation of an active transcription pre-initiation complex.

KEY WORDS: *avidin, chicken oviduct, gene regulation, inflammation, progesterone receptor*

Introduction

The early studies of Bateman (1916) on the toxicity of a raw egg-white diet in different animals led to the discovery of biotin vitamin and avidin, characterized by its high «avidity» for biotin (Eakin *et al.*, 1940; Woolley and Longsworth 1942). In the early 1940s Hertz and his co-workers (1942,1944) showed estrogen and progesterone dependency of oviductal avidin synthesis in some oviparous species. Avidin was long regarded as an oviduct and progesterone-specific protein until we (Elo *et al.*, 1975; Heinonen and Tuohimaa, 1976; Heinonen *et al.*, 1978; Elo *et al.*, 1979a) found that it can be induced without involvement of progesterone in practically all tissues except for the brain. When avidin was found in several tissues of many bird,

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amphibian and lizard species (Elo, 1980; Korpela *et al.*, 1981a, b), it could no longer be regarded as «a curiosity of the avian reproductive tract«, as claimed previously.

Because of a wide distribution of avidin in the egg-laying vertebrate species and in some microbes and because of remarkably good preservation of its function, biotin binding, in the course of evolution, an important physiological function for avidin could be expected. Since the possible functions of avidin have been reviewed in a previous report (Elo and Korpela, 1984), they are only briefly summarized in this review. The fact that avidin is present predominantly in the female reproductive tract and in the egg-white of many oviparous vertebrates (Hertz and Sebrell, 1942; Korpela *et al.*, 1981a, b) would signify a role in ovum survival and embryonic development. This may be due to three mechanisms: 1) an antimicrobial action against biotin requiring yeasts and bacteria (Board and Fuller, 1974), 2) an immunomodulatory action during the ovum passage in the oviduct (Ashorn *et al.*, 1986) and 3) metabolic effects by controlling biotin availability of biotin enzymes. The function of avidin is not restricted to reproduction, since it is found in the lung and kidney of untreated animals in certain species and in most tissues after injurious treatments (Elo, 1980). This suggests that avidin has a role in the acute defense of the host, especially because it can be induced by microbes (Elo *et al.*, 1980b). Furthermore, viral infections can cause avidin synthesis in chicken tissues or in transformed cells (Korpela *et al.*, 1982, 1983). Avidin was shown to be a physiologically functional antimicrobial system against exogenously administered microbes in the chicken peritoneal cavity (Tuohimaa, unpublished). With regard to the important functions of avidin it is rather surprising that no similar high-affinity biotin binding protein has been found in any mammalian species studied (Elo, 1980; Korpela *et al.*, 1981a, b). In fact, avidin production is lost very early in the evolution of viviparity, since the reproductive tract of the female opossum does not produce avidin in response to progestins (Tuohimaa, Niemelä and Kulomaa, unpublished).

The physico-chemical properties of avidin have also been discussed previously in many articles (see e.g. DeLange and Huang, 1971; Green, 1975); therefore they are here only briefly summarized. Avidin is a tetrameric glycoprotein with a molecular weight of 66,000-69,000. In tetrameric form each of the identical sub-units (128 amino acids, M.W. 14, 332) can bind one molecule of biotin with an extremely high affinity (Kd 10^{-15}). This property has been widely utilized methodologically by a number of authors (see for a review Bayer and Wilchek, 1980) and also by ourselves in immunohistochemistry (Isola, 1987; Isola *et al.*, 1987; Joensuu *et al.*, 1989; Pekki *et al.*, 1989), enzyme immunoassays (Vilja *et al.*, 1988) and nucleic acid hybridization techniques (Liesi *et al.*, 1987).

Because the present review deals only with a progestin-specific response of avidin gene expression, it should be emphasized that progestins potentiate or have a synergistic action on the regulation of several estrogen-specific genes such as ovalbumin, ovomucoid, conalbumin, lysozyme etc. in the chick oviduct (Schimke *et al.*, 1975; Palmiter *et al.*, 1981). Therefore, the models proposed here for the regulation of avidin production may also be applied to the regulation of other genes induced by progestins.

Avidin is induced by two different mechanisms

When we described an avidin induction by a transcription inhibitor, actinomycin D (Elo *et al.*, 1975), the meaning of this unexpected finding was not clear. At first, we thought that the induction was the so-called «superinduction» (O'Malley *et al.*, 1969) caused by small amounts of endogenous progesterone. However, we later demonstrated in detail that progesterone is not involved in the induction (Elo *et al.*, 1979a, b, 1980a), and therefore we began to call it «unspecific» or «progesterone-independent» avidin induction (Elo *et al.*, 1979b). The enigma was unravelled when we found that avidin was also induced in several tissues and by different treatments such as tissue trauma by ligation (Heinonen *et al.*, 1978), pinching or heat injury (Elo, 1980), toxic drugs (Elo *et al.*, 1975; Heinonen and Tuohimaa, 1978; Heinonen *et al.*, 1978), bacteria or viruses (Elo *et al.*, 1980b; Korpela *et al.*, 1982), all causing an inflammatory reaction (for review see Elo and Korpela, 1984). This avidin induction can be suppressed by anti-inflammatory drugs (Nordback *et al.*, 1982a; Niemelä, 1986). Therefore, as a unifying theory the notion of an «inflammation-induced» avidin production was introduced.

The inflammation-induced avidin synthesis is evidently progestin-independent (Elo *et al.*, 1979a, b). There is a wide variety of factors that cause cellular trauma associated with inflammation leading to avidin production. The response is relatively rapid and does not require tissue or cell differentiation as does progestin-dependent induction in the oviduct. Inflammation-induced avidin production appears to be local or systemic depending on the treatment (Heinonen and Tuohimaa, 1976; Elo *et al.*, 1979a, b). There is a latent period of a few hours after the trauma, during which avidin induction cannot be transferred to a culture *in vitro* (Nordback *et al.*, 1980). This might be due to the leukocyte response and production of the mediators of inflammation. Also avidin synthesis after actinomycin D administration is mediated by inflammation and it begins very slowly, in 16-24 h. Actinomycin D is a transcription inhibitor which has to be metabolized before it can induce mRNA for avidin and the synthesis of the protein. New RNA and protein synthesis is necessary for inflammation-induced avidin production (Elo *et al.*, 1981). Actinomycin D *in vitro* continuously blocks avidin production, because the compound remains almost unmetabolized in culture (Wigham *et al.*, 1980; Nordback *et al.*, 1982b). The cellular origin of inflammation-induced avidin is not yet fully characterized, but at least fibroblasts, macrophages and possibily some leukocytes appear to produce avidin (Nordback *et al.*, 1981, 1982b; Korpela *et al.*, 1983; Korpela, 1984).

The mechanism by which inflammatory agents (toxic drugs, cell injury and infection) cause the induction of avidin is not yet understood in all detail. A summary of our results depicting a model for a possible pathway of avidin induction by two different mechanisms is shown in Fig. 1. Inflammation can cause an increase in the concentration of prostaglandins, especially PG-F2α and PG-E (Heinonen *et al.*, 1978), which, in turn, can induce avidin (Heinonen, 1978; Niemelä *et al.*, 1979; Niemelä, 1986). When PG inhibitors, meclofenamic and tolfenamic acid, were used, a dose-dependent inhibition of avidin synthesis was obtained (Niemelä, 1986). This suggests that prostaglandins may act as mediators or modulators in inflammation-induced avidin production. PG effect on avidin production may be mediated by cGMP (Niemelä and Tuohimaa, 1982). The involvement of cyclic nucleotides was supported by the finding that theophylline, a potent inhibitor of phosphodiesterase, also induced avidin (Niemelä *et al.*, 1986). Cyclic GMP could act via kinases on the gene expression or directly on the 5'-flanking region of avidin gene. It will be interesting to study whether

PREs upstream from avidin genes have sequences similar to the predicted CRE (cylic nucleotide responsive element) (Mellon and Akerblom, 1989), especially because GRE/PRE (glucocorticoid or progestin responsive element) and CRE appear to be partially overlapping sequences.

The other avidin induction mechanism is clearly progestin-dependent and occurs only in the oviduct (Hertz *et al.*, 1944; O'Malley *et al.*, 1969; Tuohimaa *et al.*, 1972; Elo, 1980). The progestin-dependent avidin production is apparently mediated by the specific progestin receptors (Fig. 1). However, in chick oviduct culture hydrocortisone and prednisolone are able to induce avidin with the same potency as progesterone (Nordback *et al.*, 1982a). This can be explained by an interaction of GR (glucocorticoid receptor) and PR with the same responsive element (GRE/PRE) (Beato *et al.*, 1989; O'Malley *et al.*, 1989). Because both inflammation- and progestin-induced avidin production occur in the oviduct, it is possible that they partially overlap. The secretion of progestin-dependent avidin in particular might be mediated or modulated through the PGs induced by the stretching of the oviduct due to ovum passage. In conclusion, avidin is induced by progestin-dependent and inflammation-induced mechanisms in the oviduct and only in connection with the inflammatory reaction in the other tissues. Thus, avidin provides two interesting models to study gene regulation: 1) progestin-dependent gene regulation in the oviduct, and 2) gene regulation by the mediators of inflammation.

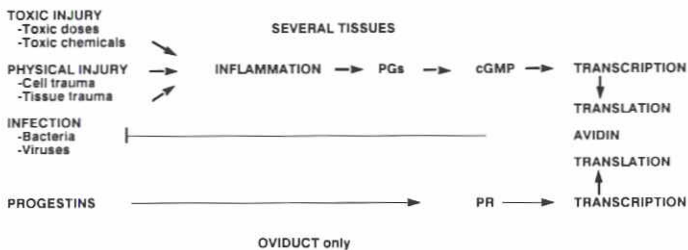

Fig. 1. *A schematic presentation of two mechanisms leading to avidin synthesis in chicken tissues. Avidin can be induced in most tissues by inflammation caused by systemic or local injurious treatments and by mediators of inflammatory reaction such as prostaglandins (PGs) and cyclic guanosine monophosphate (cGMP). A new RNA (transcription) and protein synthesis (translation) are needed for the expression of avidin. There is a closed negative feed-back control between biotin-requiring bacteria and avidin production, because avidin has an antimicrobial activity. The other avidin induction mechanism is progestin-dependent and is present only in the differentiated oviduct.*
→ = *stimulatory action,* ⊣ = *inhibitory action.*

Chicken genome contains multiple genes for avidin

Interactions of the steroid receptors and transcription factors with the gene-regulatory elements of the chicken egg-white proteins are widely used to study the regulations of eukaryotic gene expression. Avidin provides an additional model system for studies of progesterone receptor action on its specific target enhancer. The early studies suggested that progesterone regulates transcription and increases the amount of translatable avidin mRNA (Tuohimaa *et al.*, 1972; Chang *et al.*, 1973; Segal *et al.*, 1973a, b; Tuohimaa and Söderling, 1976). Our early studies also suggested some post-transcriptional regulation of avidin synthesis. (Tuohimaa and Söderling, 1977), but this needs to be restudied in detail using different RNA-hybridization analyses and avidin cDNA. Oviductal avidin production ceases during aging as an effect of the post-transcriptional regulation (Arendes *et al.*, 1980).

In order to initiate studies of avidin gene expression at the molecular level, the cDNA was isolated (Gope *et al.*, 1987). A signal peptide of 24 amino acids was found in front of the mature avidin peptide chain, giving total length of 152 amino acids and a MW of 16,815 for the preavidin molecule. The cDNA was used as a hybridization probe when induction of the avidin mRNA in the oviduct was compared to that of the avidin protein (Kunnas *et al.*, manuscript). The amount of mRNA and protein increased up to approx. 30-fold by progesterone and less than 3-fold by dexamethasone and dihydrotestosterone. No induction was seen by estradiol. There are at least two possible reasons for the unexpectedly weak effect of dexamethasone on avidin mRNA. First, dexamethasone is the least potent glucocorticoid inducing avidin *in vitro* (Nordback *et al.*, 1982a). Second, all glucocorticoids are poor inducers of avidin *in vivo* because of their rapid metabolism. Secondary stimulation by DES or estradiol did not alter the hormonal specificity. An equal induction of mRNA and protein suggest that regulation of avidin gene expression occurs mainly at the transcriptional level. Its 5'-flanking region can, therefore, be used as an experimental model to study the interaction of PR, tissue- and hormone-specific transcription factors, and gene regulatory elements in the formation of the active transcription preinitiation complex.

Two genomic clones were detected when a chicken genomic library was screened using the avidin cDNA as a hybridization probe (Keinänen *et al.*, 1988). Surprisingly, the amino acid sequence derived from the DNA sequence of the first subclone was not identical with the known sequence of egg-white avidin. As a matter of fact, the genomic clones have since been found to contain at least five «avidin-related genes», AVR1-AVR5, none of which is able to encode the known amino acid sequence of egg-white avidin. A homology of 70-85% has been observed when the four putative exons of the AVRs (Fig. 2) have been translated and compared to egg-white avidin. A unique HindIII-site is found at the 3'-end of all AVRs. Therefore, the presence of multiple genes for avidin has been confirmed by

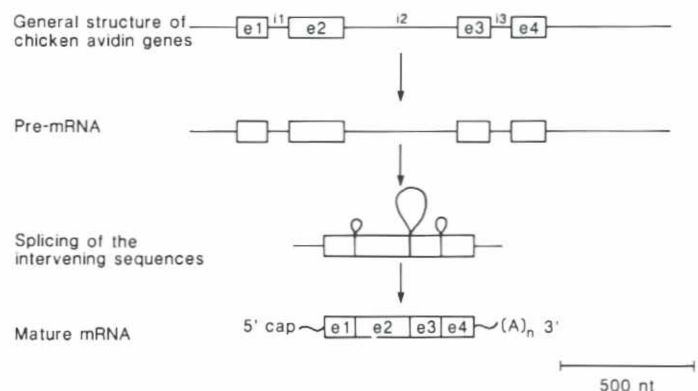

Fig. 2. *Molecular structure of avidin genes. Chicken genome contains in addition to the egg-white avidin gene at least five «avidin-related genes», AVR1-AVR5. Their general structure suggests the presence of three introns within the coding region of the genes.*

DNA hybridization analysis of the chicken genomic DNA digested with the HindIII restriction enzyme.

Avidin is also, as mentioned above, induced by a progesterone-independent induction mechanism in a number of chicken tissues in connection with tissue injury, inflammation and infections. In spite of the identical immunoreactivity and biotin-binding activity (Kulomaa *et al.*, 1981), this and egg-white avidin may not be encoded by the same gene. It is not yet known whether the AVR genes are expressed, and if so under what kind of regulation. Their possible relationship to «inflammation-induced» avidin is not known either. The avidin gene family provides, therefore, a system where a biotin-binding protein is produced either by one gene with multifactorial regulation, or by two or more closely related genes each under different regulation. Avidin and its related genes may also be used for studies of structure-function relationship of the biotin-binding proteins and their development during evolution.

Mesenchymal cells are involved in oviductal differentiation

Estrogens are the dominating factor involved in the cytodifferentiation of the immature chick oviduct (O'Malley *et al.*, 1969; Schimke *et al.*, 1975; Ratia *et al.*, 1979; Palmiter *et al.*, 1981). The first sign of the differentiation is epithelial cell proliferation (Ratia *et al.*, 1979) leading to the development of three types of epithelial cells: (1) goblet cells, (2) ciliated cells and (3) non-ciliated columnar cells. After a few days of estrogen treatment, epithelial cells begin to invaginate into the mesenchyme and to form the glands (Ratia *et al.*, 1979) which secrete ovalbumin, conalbumin, lysozyme and other estrogen-dependent secretory proteins (Fig. 3). If progesterone is administered after this primary stimulation, avidin is synthesized, whereas avidin response to progesterone is minimal in the undifferentiated oviduct (Kellokumpu-Lehtinen *et al.*, 1976; Elo *et al.* , 1979a). If progesterone is administered concomitantly with estrogen during the primary stimulation, it inhibits cytodifferentiation and gland formation (Schimke *et al.*, 1975). A single injection of estrogen with progesterone leads, however, to a further differentiation of tubular gland cells. (Joensuu, to be published). On the other hand, in the fully-differentiated oviduct progesterone induces ovalbumin and conalbumin as estrogens (secondary stimulation) (Palmiter *et al.*, 1981), but avidin expression remains specific only to progestins.

The question of the cellular origin of avidin has been a matter of conflicting results (Kohler *et al.*, 1968; Tuohimaa, 1975; Rantala *et al.*, 1982; Kami and Yasuda, 1983). Part of the problem was solved when we demonstrated that avidin is synthesized in the epithelial or glandular cells depending on the stage of differentiation (Joensuu *et al.*, 1989). We also demonstrated that goblet or ciliated cells do not produce avidin as was previously proposed (Kohler *et al.*, 1968; Rantala *et al.*, 1982). Furthermore, avidin seems to be a good marker of the differentiation of the oviduct cells (Joensuu *et al.*, 1989). However, normal differentiation was difficult to achieve by exogenous administration of estrogen if high doses of estrogen were used (Joensuu *et al.*, 1989). The mechanism of the estrogen-dependent differentiation of the progestin response in the oviduct apparently comprises the up-regulation of PR (Pekki *et al.*, 1989).

The importance of subepithelial stromal cells in the epithelial differentiation of the oviduct was recently suggested (Ylikomi *et al.*, 1987; Ylikomi and Tuohimaa, 1988). In the immature chick oviduct epithelial, submucosal mesenchyme and mesothelial (peritoneal) cells express PR in a constitutive fashion («constitutive PR», Fig. 4, left panel) or the cells are extremely sensitive to low estrogen concentrations in the blood. The first sign of oviductal differentiation can be seen in the PR negative mesenchymal cells, which begin to express PR in response to estrogen during sexual maturation or to exogenously administered estrogens. The estrogen-inducible PR in the mesenchymal and smooth muscle cells is called «inducible PR» (Fig. 4, middle panel). Gradually the PR-expressing mesenchymal cells invade mucosal plicae and once they come into close contact with the epithelium, it begins to form glands, suggesting an «interaction between mesenchymal and epithelial cells» in glandular differentiation. Stimulation with estrogens causes a layer of PR-negative cells (luminal nuclei) in the epithelium of the oviduct (Joensuu *et al.*, 1989), which appear to be mitotic or ovalbumin-producing cells, but they do not produce avidin.

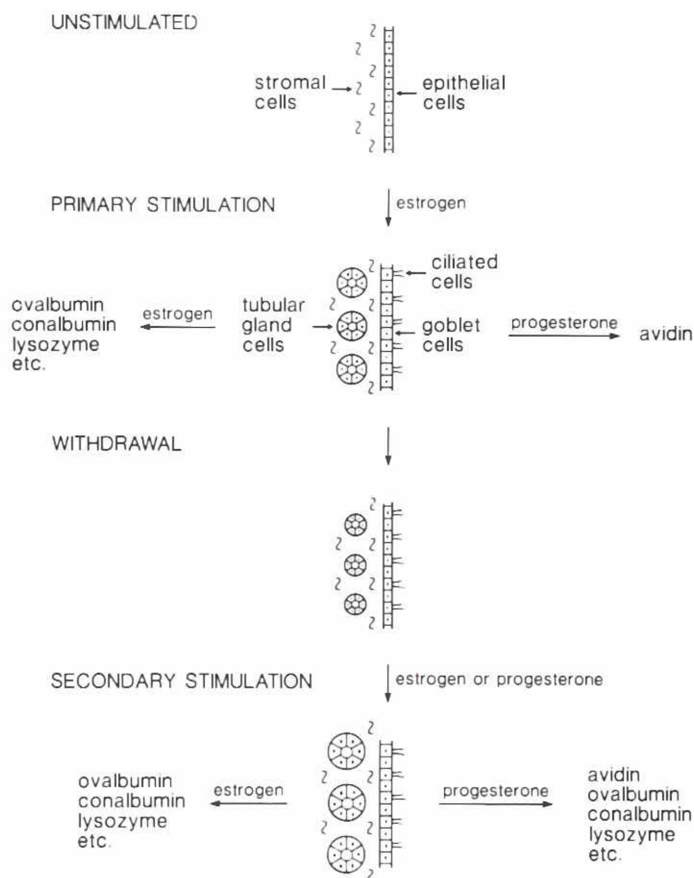

Fig. 3 *Hormonal differentiation of the immature chick oviduct can be caused by multiple estrogen administration (primary stimulation) leading to development of estrogen-responsive tubular glands and to progestin responsiveness. After a withdrawal of estrogen for several days the synthesis of specific proteins can be reinitiated by a single injection of estrogen or progesterone (secondary stimulation).*

Progestin response of the differentiated oviduct is characterized by a decrease in PR immunoreactivity (Pekki *et al.*, 1989; Joensuu *et al.*, manuscript). The «down-regulation» occurs in all the cell types expressing PR. It begins at 2 h after progesterone administration and PR is back to its original level at about

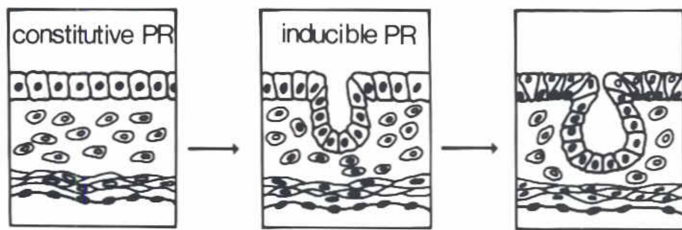

Fig. 4. *Appearance of constitutive and inducible progesterone receptor (PR) during the differentiation of the oviduct with estrogen. In the immature chick oviduct without exogenous or endogenous estrogens epithelial and peritoneal cells express PR constitutively (left panel). When the chicks are treated with estrogen or are sexually maturing, stromal and smooth muscle cells begin to express inducible PR (middle panel). After secondary stimulation with estrogen a layer of PR-negative epithelial cells appears and the antigenic activity of PR in most of the cells decreases (right panel).*

1 day after a single injection of progesterone. This correlates well with the known transcriptional activation of avidin and other progesterone-inducible genes. However the significance of the «down-regulation» of PR is not understood. It may reflect a new protein-protein or protein-DNA interaction, or a new chromatin organization leading to occult antigenic determinants, or simply receptor degradation. Progestins appear to increase PR degradation and to decrease PR synthesis in human breast cancer cells (MCF-7) (Nardulli *et al.*, 1988). It is remarkable that the most prominent «down-regulation» of PR is observed in the «inducible PR» found in the mesenchymal cells, since practically all the antigenic activity of PR disappears after progesterone occupation. The «down-regulation» of PR seems to be steroid-specific, since at best a weak «down-regulation» of PR also occurred after estrogen or cyproterone acetate treatments, i.e. with ligands not binding to PR (Pekki *et al.*, 1989; Joensuu *et al.*, to be published). Therefore, the «specific down-regulation» of PR may be a result of a conformational change of chromatin and it is associated with progestin-specific response.

Nuclear progesterone receptor may act in one step

Two forms A (72K) and B (86K) of PR have been observed in the chicken oviduct. They have the same amino acid sequence except that A is N-terminally truncated (Renoir *et al.*, 1983; Jeltsch *et al.*, 1986; Conneely *et al.*, 1987; Gronemeyer *et al.*, 1987). PR is estrogen-inducible, which may be part of the oviduct differentiation. Also, progestins are able to induce their own receptor (Ylikomi *et al.*, 1984). The predicted functional domains of PR (ligand binding, intracellular localization, DNA binding by two zinc fingers, dimerization, binding of transcription and transcription activation) are at the moment only partially characterized (Tora *et al.*, 1988; Gronemeyer *et al.*, 1989; O'Malley *et al.*, 1989).

The unoccupied PR sediments in sucrose gradient ultracentrifugation at 8S, which has been shown to contain one molecule of PR A or B and two molecules of heat shock protein 90 (hsp90), which is common to all steroid receptors (Baulieu *et al.*, 1983; Joab *et al.*, 1984). The 8S complex is formed in the cytosol when tissues are homogenized in a buffer with low ionic strength. 8S PR can be activated by elevated temperatures, ligand occupation or high salt concentrations to 4S-form containing only single binding components A or B (Baulieu *et al.*, 1983). 8S-4S transformation was called activation, and for about 20 years it was thought to occur in the cytoplasm. Because PR occupied with ligand is found in the nuclear fraction, it was evident that ligand causes a nuclear translocation and binding to DNA («two-step model») (O'Malley *et al.*, 1970). Studies on the structure and location of different PR forms were not possible before the first specific antibodies against purified PR were available (Tuohimaa *et al.*, 1984b).

Using the antibodies specific for all the binding forms of PR including the microsomal PR (Haukkamaa *et al.*, 1980) and the smallest binding «mero» form (Tuohimaa *et al.*, 1984a), immunohistochemical studies showed that all ligand binding forms of PR locate inside the nucleus of target organs (Gasc *et al.*, 1984; Isola *et al.*, 1986, 1987; Isola, 1987; Ylikomi *et al.*, 1987). Highly sensitive immunoelectron microscopy detected no cytoplasmic PR except for its synthesis in the rough endoplasmic reticulum near nuclear pores of a few cells. Our recent immunobiochemical studies suggested that the microsomal PR might also be a contamination of cytosol with nuclear PR (Tuohimaa *et al.*, 1988). Therefore we concluded that there is no functional cytoplasmic or microsomal PR, and that PR is entirely a nuclear protein. This was further verified by a new immunohistochemical technique (freeze-drying and vapor fixation), which eliminates the possible diffusion of soluble proteins (Pekki and Tuohimaa, 1989).

According to biochemical data the PR-hsp90 complex is unable to bind to DNA and is thus transcriptionally inactive (Baulieu, 1987). According to this model steroid occupation causes a dissociation of the receptor-hsp90 complex and the receptor is translocated to the nuclei or transformed inside them. As indicated above, the unoccupied PR is exclusively nuclear protein, whereas hsp90 is localized in the cytoplasm (Tuohimaa *et al.*, 1989) (Fig. 5). Because the sensitivity of our immunohistochemical method might be insufficient to demonstrate possible small nuclear concentrations of hsp90, we used a PR-antibody recognizing an epitope, which is occult in the PR-hsp90 complex. With this antibody a nuclear location of an unoccupied PR was achieved similar to that detected with the other receptor antibodies, indicating that PR is not bound to hsp90 in the nucleus of intact cells (Pekki *et al.*, manuscript). Thus, the PR-hsp90 complex is probably formed during homogenization. In fact, our results would indicate that the intranuclear PR consists of binding components of PR (A or B) only, which are apparently in a DNA binding state (O'Malley *et al.*, 1989). Although our results suggest that hsp90 is not involved in the intranuclear regulation of the action of PR, it is not excluded that hsp90 has a functional role in the cytoplasm such as transportation of newly synthesized PR to the nucleus.

When the intranuclear location of PR was studied in detail by electron microscopy using the immunogold technique, it was found that the unoccupied PR was preferentially located on the condensed chromatin (Fig. 6) (Isola 1987). After occupation the ration of heterochromatin/euchromatin decreased and the PR was mostly seen over the dispersed chromatin, mainly in the marginal area between condensed and dispersed chromatin (Fig 7). This is apparently the active transcription site. The decrease in the relative amount of heterochromatin (Kellokumpu-Lehtinen *et al.*, 1976) probably reflects the decondensation of chromatin required for the initiation of transcription.

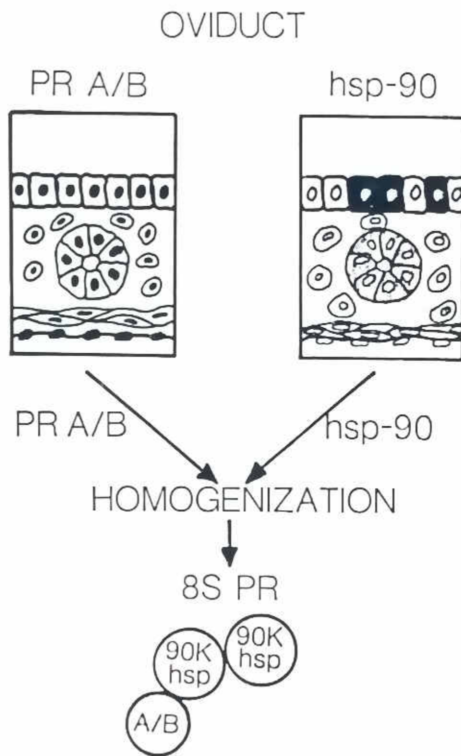

Fig. 5. *Components of the 8S form of PR, ligand-binding components A or B and heat shock protein 90 (= hsp90) are located in different cellular and subcellular compartments. Therefore the 8S form of PR, which contains one molecule of PR A or B and two molecules of hsp90, is possibly a homogenization artefact not present in intact target cells. Black staining indicates the localization of PR and hsp90, respectively. PR is localized in the nuclei of all cell types, whereas the highest concentration of hsp90 is found in the cytoplasm of some epithelial cells and less in the cytoplasm of other cells.*

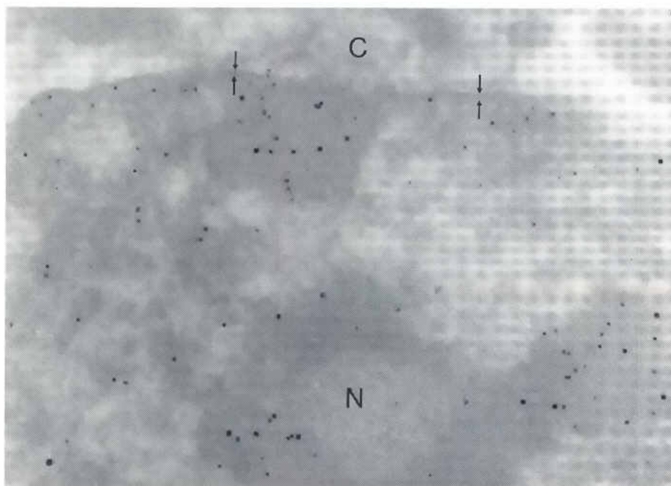

Fig. 6. *Immunoelectron-microscopic location of unoccupied chicken PR on the heterochromatin by immunogold staining. Arrows point to the nuclear envelope. All PR is located inside the nucleus and there is no PR in the cytoplasm (= C). N = nucleolus. Magnification 45,000 x.*

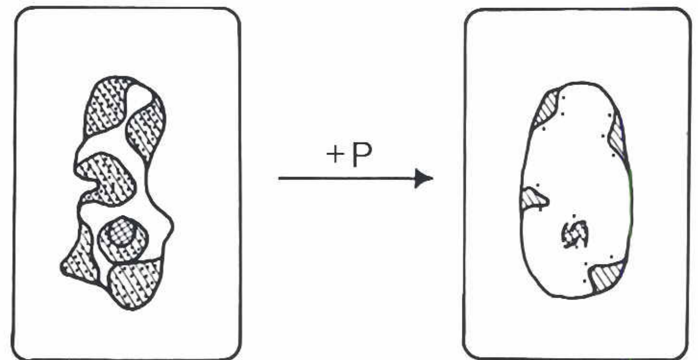

Fig. 7. *A schematic presentation of the effect of ligand occupation on the intranuclear localization of PR and on chromatin change. The unoccupied PR is located mainly on the heterochromatin, which is partially changed to euchromatin by progesterone. Some of the antigenic reactivity of PR disappears after ligand occupation, and the rest can be visualized at the margins of the heterochromatin.*

Several phenomena have been proposed as following the occupation of PR with the ligand: (1) The first (N-terminal) zinc finger of PR is more tightly bound to the guanine residue of the major groove of DNA (Tora *et al.*, 1988; O'Malley *et al.*, 1989). The tight binding of the receptor in the nuclei after hormone occupation is probably mostly due to the change in the receptor conformation induced by ligand binding (Fig. 8) and to a lesser extent to the exposition of PRE, because the phenomenon is also seen in the nontarget cells transfected by different

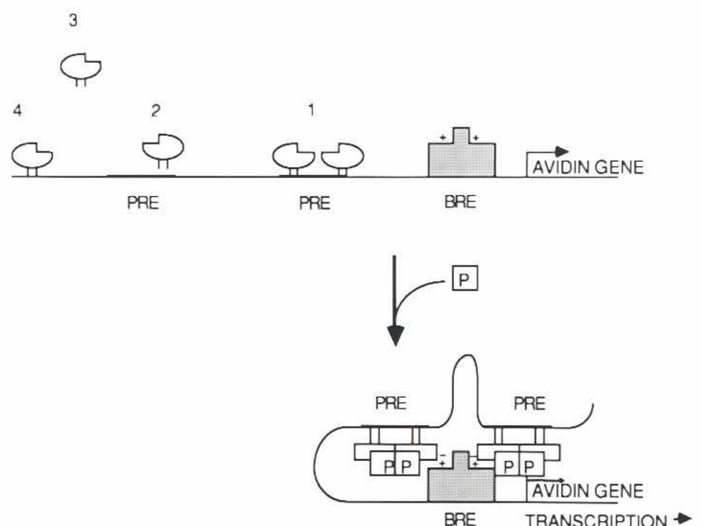

Fig. 8. *A hypothetical «one-step-model» of PR action at the 5'-flanking region of a progestin-responsive gene. The occupation of PR with ligand leads to formation of PR dimers in head-to-head orientation and to a conformational change of PR and formation of the transcription pre-initiation complex with transcription factors (black box) at the basic regulatory element (BRE) and to a looping of DNA. This is observed in the immunohistochemistry as a decrease in the antigenic activity of PR. There are at least 4 possible ways in which the unoccupied PR might be associated with DNA: (1) PR is directly bound to the progesterone regulatory element (PRE). (2) PR is loosely bound at or near PRE. (3) PR is in the heterochromatin, but is indirectly bound to DNA (via an acceptor for PR?). (4) PR is bound to nonspecific DNA sequences.*

steroid receptors (2). The receptors form dimers by protein-protein interaction (Kumar and Chambon, 1988; O'Malley *et al.*, 1989). (3) PR appears to be phosphorylated (García *et al.*, 1983, 1984) probably in ligand-dependent manner (Logeat *et al.*, 1985). (4) A transactivation through the A/B-domain is initiated (Tora *et al.*, 1988; Gronemeyer *et al.*, 1989). This process probably involves an interaction between the receptor and transcription factors (Fig. 8). (5) Proteolysis of PR is enhanced (Sullivan *et al.*, 1988) and synthesis of its own mRNA is repressed (Nardulli *et al.*, 1988). (6) Chromatin structure is rapidly changed by progesterone. This reversible chromatin change is different from the chromatin change during the estrogen-induced differentiation (Kellokumpu-Lehtinen *et al.*, 1976; O'Malley *et al.*, 1983). (7) PR and the transcribed genes might be associated with nuclear matrix (O'Malley *et al.*, 1983). All these changes are reflected as a decrease in immunoreactive PR in the histochemistry (Pekki *et al.*, 1989) which («down-regulation») appears to be closely associated with gene activation. The new intranuclear location of PR at the margins of heterochromatin after ligand occupation does not necessarily mean an intranuclear translocation of the receptor, for it may be due to a decondensation of the chromatin at the receptor binding site (Fig. 7). In summary it can be said that both the unoccupied and occupied PR are located close to the site of its action. Therefore we propose a «one-step-model» of PR action which does not involve 8S-4S transformation or translocation processes.

Gene regulation by progesterone receptor

Expression of progestin-inducible genes requires two phases. *First*, cell differentiation is associated with a new «irreversible» organization of the chromatin (O'Malley *et al.*, 1983), which renders the regulatory elements of the gene accessible for transcription factors. This is characterized by an appearance of DNase I-hypersensitive regions (Kaye *et al.*, 1986) and is mainly an effect of estrogens. The synthesis of PR in the epithelial and mesenchymal cells appears to be important for this phase of differentiation (Ylikomi *et al.*, 1988). *Second*, binding of PR to PRE (progesterone responsive element) and to the transcription factors, and formation of a transcription preinitiation complex at the promoter region (Ptashne, 1988). After formation of the complex, RNA-polymerase can bind and begin RNA synthesis. Also this phase of differentiation is characterized by a new but reversible organization of the chromatin. The location of SREs (steroid responsive elements) varies from gene to gene. They may be several kilobases upstream from the promoter or a few hundred base pairs upstream or downstream from the transcription initiation site (Beato, 1985; Cato *et al.*, 1986). If SRE is positioned immediately upstream from the TATA box, a single SRE is able to form a steroid-inducible transcription complex (Strähle *et al.*, 1988). Two SREs are needed for the initiation of transcription if SRE is at a position further upstream. The DNA sequences (SRE) required for the induction by glucocorticoid (GR), progestin (PR), androgen (AR), estrogen (ER) or ecdysone receptors are either identical or closely related (Strähle *et al.*, 1988). It is known that the progesterone-responsive element (PRE) is similar or even identical to the glucocorticoid-responsive element (GRE) (Ahe *et al.*, 1985). Therefore, it is not surprising that we have obtained similar effects on avidin synthesis by progesterone and glucocorticoids *in vitro* (Nordback *et al.*, 1982a). The basic element of GRE/PRE is a 15-mer palindromic structure composed of two conserved hexanucleotide motifs (TGTTCT) separted by 3 base pairs (Beato *et al.*, 1989; O'Malley *et al.*, 1989). It has been proposed that amino terminal zinc-finger of a receptor monomer appears to interact with the major groove of each hexanucleotide motif leading to the formation of a receptor dimer in head-to-head orientation, and it determines the specificity of the receptor action (Green and Chambon, 1987; Kumar and Chambon, 1988; Tora *et al.*, 1988; Beato *et al.*, 1989; O'Malley *et al.*, 1989). The carboxy terminal zinc-finger contacts the DNA backbone flanking the 15-mer in a base-independent manner, and contributes to the free energy of binding but not sequence recognition (Beato *et al.*, 1989).

Cell specificity seems not to be determined exclusively by the receptor and SRE. Our results indicate that all the cells producing progestin-inducible proteins contain PR, but several PR-positive cells (epithelial, mesenchymal, smooth muscle, peritoneal and neuronal cells) do not produce avidin or other secretory proteins in response to progestins (Joensuu *et al.*, 1989), although at least mesenchymal cells have a capacity to produce avidin in response to trauma (Nordback *et al.*, 1981). Therefore, it can be concluded that other transcription factors are also required in addition to steroid receptors to define the cellular specificity of the transcription (Dierich *et al.*, 1987).

It is not known how the unoccupied receptors are associated within the nuclei. Being ligand-free they are easily extractable from the nuclei, but after occupation with the ligand *in vivo* they are bound more tightly, although there is relatively little difference in the affinity. On the other hand, it has been shown that unoccupied receptor is able to bind to the progesterone regulatory element (PRE) *in vitro* (Willmann and Beato, 1986; O'Malley *et al.*, 1989). Genomic footprinting *in vivo* has failed to demonstrate that the PR is bound to PRE unless the receptor is occupied by the ligand (Becker *et al.*, 1986). Thus, it is probable that unoccupied receptors, as charged molecules, are mainly non-specifically bound to DNA (Fig. 8, alternative 4) and chromatin proteins (Fig. 8, alternative 3). On the other hand, it has been shown that PR without ligand can also act as a repressor of specific inhibitory elements (Gaub *et al.*, 1987). Thus, it is possible that unoccupied PR is also located at the specific regulatory sequences (Fig. 8, alternatives 1 and 2). This would fit with our immunoelectron-microscopic findings suggesting that PR is not randomly localized but mainly localized on the heterochromatin (Isola, 1987) (Fig. 6). In fact, the off-rate of steroid receptor from DNA was accelerated in consequence of ligand binding, whereas the on-rate was not significantly changed (Beato *et al.*, 1989). This would indicate a more rigid binding of the unoccupied receptor to DNA than after occupation. Furthermore, different forms of PR (A and B) might have different or even oppposite actions on gene regulation (Tora *et al.*, 1988; Gronemeyer *et al.*, 1989).

In conclusion, Fig. 8 shows a hypothetical model of the interaction of PR with PRE and with general and tissue- or hormone-specific transcription factors, i.e. the formation of a steroid-inducible transcription pre-initiation complex. The transcription initiation factors located in the basal regulatory element (BRE) are complexed or regulated by occupied PR dimers. When two PREs are present, a looping of intervening DNA sequences can be expected (Theveny *et al.*, 1987). This is possibly how distant regulatory elements are able to control promoter activity. This is probably carried out by protein-protein interaction between the receptor and the proteins at the basal regulatory element (Ptashne, 1988). Whether the interaction is direct or mediated by other regulatory proteins is not known.

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