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Subcellular Distribution of Estrogen Receptor and Progesterone Receptor with and Without Specific Ligand

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The estrogen receptor (ER) and progesterone receptor (PR) content of cultured human breast carcinoma cells (MCF-7) was determined by biochemical assay, immunoblot analysis, and immunohistochemical assay under varying conditions of hormonal stimulation. The ER and PR content in cytosolic and nuclear extracts varied with steroid treatment. However, both the amount and distribution of each receptor in these extracts was virtually the same when determined by steroid binding and immunoblot analyses. Two immunocytochemical parameters (staining intensity and proportion of cells stained) correlated with the quantitative analyses of ER and PR, but not with the subcellular distribution. When MCF-7 cells were grown for 4 days in charcoal-stripped serum without phenol red, 93% of total ER was found in the cytosol (10 mM KCl), whereas short-term treatment with 5 nM estradiol resulted in the appearance of 82% of total ER in the nuclear extract (400 mM KCl). With either cell treatment only nuclear staining for ER was observed. Progesterone receptor was virtually undetectable in the same cells by any method. After 4 days of treatment by 5 nM estradiol, PR was strongly induced (50-fold) in MCF-7 cells as determined by all three methods. As observed for ER, 95% of total induced PR was found in the cytosol in the absence of a progestin. Short-term treatment with 5 nM ORG 2058, a synthetic progestin, resulted in the appearance of 42% of total PR in the nuclear extract. However, only strong nuclear staining for PR was observed in either the presence or absence of a progestin. These findings are consistent with the current view of ER and PR as nuclear receptors present in at least two forms. One of these, the unoccupied form of the receptor, is easily removed from the nucleus by hypotonic buffers during the cell homogenization process and appears in the cytosolic extract. The other form of the receptor, the steroid-occupied form, is more tightly bound to nuclear components and is removed from nuclei only under more vigorous extraction conditions. (Am J Pathol 1989, 135:857–864)

Immunohistochemical observations using monoclonal receptor antibodies[1–3] and cellular enucleation studies[4,5] suggested that both the steroid-occupied and unoccupied forms of ER and PR are located in the nucleus of intact responsive cells. Before the publication of these studies most investigators accepted a model of ER and PR action in which the unoccupied form of the receptor, located in the cytoplasm of the cell, became activated after binding steroid hormone and was translocated to the nucleus where most of the steroid-occupied form of the receptor resided.[6–9] The experimental data supporting this model was based largely on the ease of extraction of unoccupied receptor from tissue homogenates by hypotonic buffers and the identification of ER and PR in the cytosolic extracts with radiolabeled ligands.

We analyzed the receptor content of cultured human breast carcinoma cells (MCF-7), an ER-rich cell line in which PR is induced by estrogen treatment.[10,11] The ER and PR distribution each was characterized by three different methods, in both the presence and absence of specific ligand. Immunohistochemical staining patterns were compared with the results of conventional ligand-binding assays and western immunoblots. The data suggested that virtually all of the immunoreactive receptor is present in the nuclei of responsive cells, regardless of the distribution of ER and PR between cytosolic and nuclear extracts.

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Materials and Methods

Cultured Cells

Three groups of four T-150 flasks, each containing two sterile glass slides, were seeded with human breast carcinoma cells (MCF-7). The cells were grown for 3 days in phenol red-containing Eagle's minimal essential medium and then, because of the known estrogenic effect of phenol red,[11] were transferred to phenol red-free medium for 4 days. The Minimum Essential Medium (Eagle) was supplemented with Hank's salts, L-glutamine, nonessential amino acids (GIBCO Laboratories, Grand Island, NY), insulin (5 μg/ml), sodium pyruvate (110 μg/ml), sodium bicarbonate (0.075%), and heat-inactivated, charcoal-stripped calf serum (5%).[12] During the 1st week medium was changed every 2nd day[13]; subsequently, the medium was changed daily. After the 1st week each set of four flasks was treated separately as follows with the culture medium changed daily: 1) One flask was maintained in phenol red-free medium for 4 more days without steroid treatment; 2) a second flask, maintained in phenol red-free medium for 4 additional days, was treated with 17 β-estradiol ([^{3}H]-estradiol, 5 nM, 57 Ci/mmol, 115,000 dpm/ml) half an hour before harvesting the cells; 3) a third flask was treated for 4 days with estradiol-containing (5 nM) phenol red-free medium to induce PR production by the MCF-7 cells, which were harvested without progestin treatment; and 4) the fourth flask received estradiol-containing (5 nM) phenol red-free medium for 4 days and then a progestin, ORG2058 (16 alpha-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione), was added one half hour before harvesting the cells.

The glass microscope slides were removed from each flask and processed for immunohistochemical localization of ER and PR. The remaining cells in the flask were removed with a rubber policeman and processed for a biochemical assay and an immunoblot analysis of receptor content.

Immunocytochemical Localization of Receptor

MCF-7 cells on glass slides were fixed for 5 minutes in picric acid-paraformaldehyde fixative[14] and immunostained by the peroxidase–antiperoxidase technique[15] using monoclonal ER antibodies (H226 or H222)[16,17] or monoclonal PR antibodies (JZB39 or KD68),[18–20] as described elsewhere in detail.[20,21] The microscope slides with MCF-7 cells on them were carefully divided into two parts with monoclonal receptor antibody incubated on half of the slide and control antibody (normal rat IgG) incubated on the other half of the slide. Incubation with the rat monoclonal receptor antibodies (10 μg/ml) or control rat immunoglobulin (10 μg/ml) was followed by treatment with a goat anti-Lewis rat IgG "bridging" antibody and rat peroxidase antiperoxidase complex (Sternberger-Meyer, Inc.). Each antibody incubation was followed by three 5-minute washes in phosphate-buffered saline. The chromogen was 3-3′ diaminobenzidine.[15] All monoclonal receptor antibodies used in this study were derived from male Lewis rats immunized with either partially purified MCF-7 cytosolic ER[16,17] or partially purified PR derived from T47D human breast carcinoma cells.[18] These antibodies specifically recognize both the steroid-occupied and unoccupied forms of either ER or PR.[18,22,23]

Preparation of Cytosolic and Nuclear Extracts

Cells released from T-150 flasks by scraping with a rubber policeman were collected by centrifugation at 1000*g* and washed with two portions of ice-cold 10 mM Tris buffer, pH 7.4. The cells were then resuspended in four volumes of cytosol buffer (10 mM Tris, pH 7.4 containing 20 mM sodium molybdate and 1 mM dithiothreitol) and homogenized with a Polytron PT-10 homogenizer in an ice bath. A cytosol fraction was prepared by repeated vortexing of the suspension at 4 C for 30 minutes, followed by centrifugation of the homogenate at 13,000*g* for 30 minutes to remove nuclei and cell debris. The crude nuclear pellet was washed twice with Tris buffer minus molybdate and then extracted with four volumes of 10-mM Tris buffer, pH 7.4, containing 600 mM KCl. The suspension was centrifuged at 13,000*g* for 30 minutes to give a clarified nuclear extract. The residual nuclear pellet was extracted again with SDS containing sample buffer and pooled separately as "pellet."

Extracts of cells that had been incubated in medium containing 5 nM E* (6.2 Ci/mmol) were analyzed directly on controlled-pore glass bead (CPG) columns, whereas extracts of tissue incubated in steroid-free media were first labeled with 5 nM E* (57 Ci/mmol) overnight at 0 to 2 C.

Biochemical Assay of Receptor Content (Controlled-Pore Glass Bead Assay)

The ER content of each extract was measured by the specific binding of E*R to CPG beads, as described elsewhere[24–26]; nonspecific E* binding was determined by heating labeled samples at 60 C for 15 minutes to destroy E*R complexes. Briefly, 100-μl aliquots were applied to 0.2-ml columns of CPG beads at 4 C. Each column was washed with 20 ml of 10 mM Tris buffer, pH 7.4, containing 400 mM KCl. Bound E* was eluted at room temperature with two 1-ml portions of absolute ethanol, and radioactivity was measured in a toluene-based scintillation mix-

Table 1. *Distribution of ER and PR in MCF-7 Human Breast Carcinoma Extracts*

Treatment	ER content		Progestin Receptor content	
	Cytosol	Nuclear extract	Cytosol	Nuclear extract
No steroid	2.76 (93%)	0.22 (7%)	0.12	0.05
E_2 30 minutes	0.48 (18%)	2.24 (82%)	—	—
E_2 4 days	0.20	—	8.12 (95%)	0.40 (5%)
E_2 + ORG†	—	—	4.32 (58%)	3.16 (42%)

† ER and progestin receptor content expressed in picomoles of estrogen receptor per gram tissue weight.

ture (10 ml per sample) at 40% counting efficiency. All radioactivity measurements were corrected for quenching by ethanol.

PR content was similarly determined using [^{3}H]ORG-2058 (16 alpha-ethyl-21-hydroxy-19-nor-pregn-4-ene-3, 20-dione,52 Ci/mmol) instead of estradiol as the ligand.

Western Immunoblot Analysis

Western immunoblots were prepared as described.[18,27] Cytosolic extracts, nuclear extracts, and extracts from the residual pellet were analyzed by electrophoresis under reducing conditions in 10% polyacrylamide slab gels. Before electrophoresis, samples were diluted directly in sample buffer (50 mM Tris, pH 6.8, 10% sucrose, 2% sodium dodecyl sulfate [SDS], 5% beta-mercaptoethanol, and 0.005% bromphenol blue) and heated at 100 C for 5 minutes. Aliquots (50 μl/well) of each sample, or marker proteins, were applied to the wells of a 7% polyacrylamide stacking gel, and electrophoresis was carried out at 4 C overnight at 10 mA. Molecular weight standards were as follows: myosin M, 200,000; beta-galactosidase M, 116,000; phosphorylase B M, 97,400; bovine serum albumin M, 67,000; ovalbumin M, 45,000; carbonic anhydrase M, 30,000; and lysozyme M, 14,400 (Bio-Rad). Proteins were transferred from SDS gels to nitrocellulose by electrophoresis at 4 C and 0.90 amperes for 2 hours in a buffer containing 0.025 M glycine, 0.192 M Tris, and 20% methanol.[27] Lanes containing molecular weight standards were cut out and stained with Ponceau S according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). After transfer from the SDS gel to a nitrocellulose filter, nonspecific binding to the nitrocellulose was reduced by treating the nitrocellulose with filtered 3% milk (Carnation Skim) in tris-saline buffer (50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.2% Tween 20 for 1 hour. The filters were drained but not washed and incubated with the following solutions for 1 hour each at room temperature: primary antibody solution (2 to 4 μg monoclonal ER or PR antibody/ml in 1% milk in TBS/0.2% Tween 20), bridging antibody (rabbit anti-rat IgG, 2 μg/ml in incubation buffer), and ^{125}I-Protein A (1×10^6 CPM/ml in incubation buffer). After each incubation the filters were drained and washed twice for 5 minutes in TBS/0.2% Tween 20 buffer. The nitrocellulose filters were then air-dried and placed in cassettes for varying periods of time with film to produce autoradiograms of the filters.

Results

Distribution of ER

Cultured MCF-7 human breast carcinoma cells, incubated in tissue culture medium without added estradiol, had most of the ER in the cytosolic extract (93%) and only a fraction of the ER in the nuclear extract (7%) by both biochemical assay (Table 1) and immunoblot analysis (Figure 1A). After short-term treatment (half hour) with estradiol-containing medium, most of the ER was present in the nuclear extract (82%), consistent with the classical concept of receptor "translocation" from the cytosol to the nuclear extract. Only a small amount of ER (18%) remained in the cytosolic extract (Table 1) and a minimal amount of unextracted ER was present in the residual pellet (Figure 1A).

In contrast, the immunocytochemical localization of ER showed only nuclear staining with either short-term estradiol treatment or no estradiol treatment (Figure 1B–D). No specific cytoplasmic immunostaining was observed. Approximately 90% of the cells showed nuclear immunostaining for ER (Table 2). The intensity of nuclear immunostaining for ER was slightly stronger with no treatment than with short-term estradiol treatment, although it was occasionally of comparable intensity (compare Figure 1B with 1C).

Distribution of PR

The amount of expressed PR was very low in MCF-7 cells grown in phenol red-free medium with charcoal-stripped serum and no added estradiol. Such uninduced cells had little or no detectable PR by steroid-binding assay (Table 1) and western immunoblot analysis (Figure 2A) and only a few PR-immunoreactive cells by immunocytochemistry (Figure 2B). Treatment of these MCF-7 cells with estradiol-containing (5 nM) medium for 4 days resulted in a substantial (50-fold) induction of PR protein identified with all

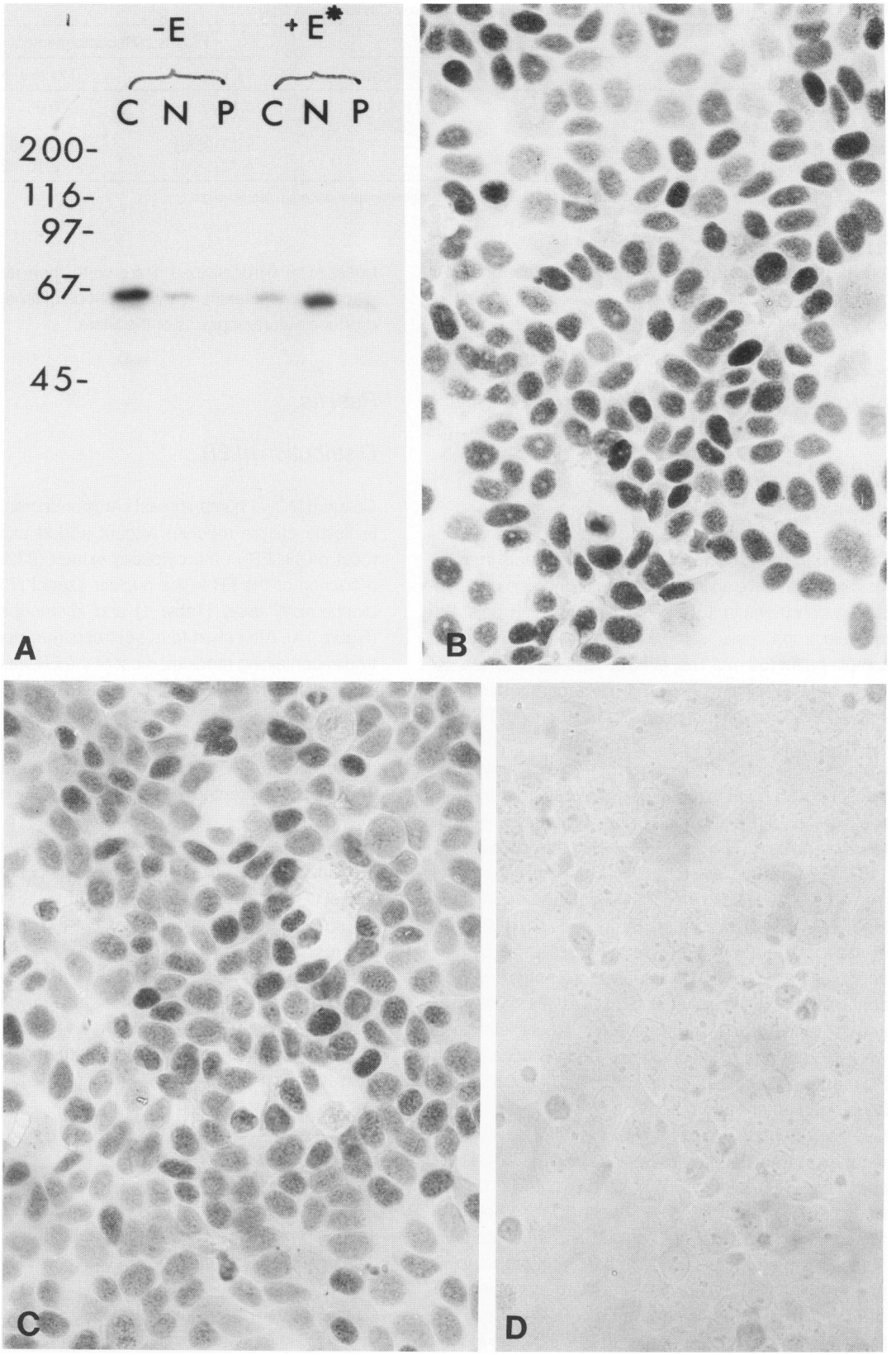
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Figure 1. *Identification of ER with monoclonal antibody.* A: *Immunoblot analysis of cytosolic extract (C), nuclear extract (N), and residual pellet (P) from MCF-7 cells that either received no estradiol (−E) or were treated with short-term estradiol (5 nM, 30 minutes) (+E*).* B–D: *Immunohistochemical localization of ER in MCF-7 cells. Exclusively nuclear localization of estrogen receptor was obtained either without* (B) *or with* (C) *estradiol added to the culture medium 30 minutes previously. The intensity of nuclear staining in both* B *and* C *varies in individual cells from weak to intense with nearly all of the nuclei showing at least weak immunostaining. Negative controls* (D) *treated with normal rat IgG instead of rat monoclonal ER antibody showed only nonspecific staining (no counterstain, ×400).*

three assays (Table 1, Figure 2A, D, E). These observations are consistent with the regulation of both PR protein and PR messenger RNA levels by estrogen described by others.[28,29] With no short-term progestin treatment, both the biochemical assay and immunoblot analysis demonstrated most of the receptor in the cytosolic extract (95%), only a small amount of receptor in the nuclear extract (5%), and undetectable levels in the residual pellet (Figure 2A). However, after short-term progestin treatment much of the PR was identified in the nuclear extract (42%) with approximately half of the PR in the cytosolic extract (58%) (Table 1 and Figure 2A), and only minute amounts of PR detectable in the residual pellet (Figure 2A).

Immunohistochemical localization of PR both with and without short-term progestin treatment demonstrated an exclusively nuclear distribution of receptor (Figure 2D, 2E). No specific cytoplasmic immunostaining for PR was observed in these cells. PR, present in less than 5% of the cells before estradiol induction, was identified in approximately 90% of the MCF-7 cells after estradiol induction of PR both with and without short-term progestin treatment (Table 2).

Discussion

The production of monoclonal antibodies to ER and PR has made the development of new assays for ER and PR possible. However, each of these assays provides a slightly different measure of receptor. We considered it important to characterize ER and PR proteins in a controlled model system comparing receptor data derived from antibody based assays with data derived from ligand-binding assays. The MCF-7 human breast cancer cell line contains high levels of ER and an estrogen-inducible PR[10,28,29] and therefore was considered to be ideal for this comparison. The antibodies used here to identify ER (H222 and H226) and PR (JZB39 and KD68) in the western immunoblot analysis and immunohistochemical assay were shown to recognize both the occupied and unoccupied forms of the receptors with equal facility.[18,22,30] The distribution of receptors determined with the conventional steroid-binding assay and the immunoblot assay was very similar although one of these assays is based on the ability of radiolabeled ligand to bind to the receptor and the other is based on the ability of a monoclonal antibody to recognize receptor. The immunoblots confirmed that nearly all of the receptor is removed from the cells by the extraction procedures, with only small amounts of ER and PR remaining in the residual cell pellets after extraction is completed.

The finding of exclusively nuclear localization of receptors by immunohistochemistry, in contrast to their observed abundance in cytosolic extracts analyzed by both antibody-based and radioligand-based methods, raised the possibility that a cytoplasmic form of receptor may be selectively lost by immunohistochemical tissue processing. If this were the case, based on the results of cytosolic extracts, we would predict an increase in nuclear immunostaining after short-term treatment with steroid. A previously unidentified "cytoplasmic receptor" would appear in the nucleus after "translocation". This was not observed. In fact, the slight reduction in nuclear immunostaining after steroid treatment was in agreement with other studies reporting a reduction in ER messenger RNA and PR messenger RNA, as well as a reduction in protein product after steroid treatment.[28,29,31] Although from 25% to 60% of the total ER content of unfixed, frozen sections can be removed by incubation in buffer at room temperature,[32,33] most radiolabeled receptor (approximately 90%) layered onto lyophilized tissue sections remained bound to the tissue section after fixation with aldehyde-contain-

Table 2. *Percentage of MCF-7 Cells Stained for ER and PR by Immunocytochemistry*†

Treatment	Cells with immunostaining for ER‡				Cells with immunostaining for PR‡			
	Intense	Moderate	Weak	Negative	Intense	Moderate	Weak	Negative
No treatment	50	29	16	5	1	2	1	96
E_2 30 minutes	5	31	46	18	4	6	7	83
E_2 4 days	0	4	56	40	34	43	15	8
E_2 + ORG*	1	13	50	36	39	42	9	10

† At least 200 cells were counted. Intense, intense nuclear staining; moderate, strong nuclear staining but not as strong as intense; weak, distinct but weak nuclear staining; negative, no nuclear staining.

‡ Only nuclear immunostaining was identified; no specific cytoplasmic immunostaining.

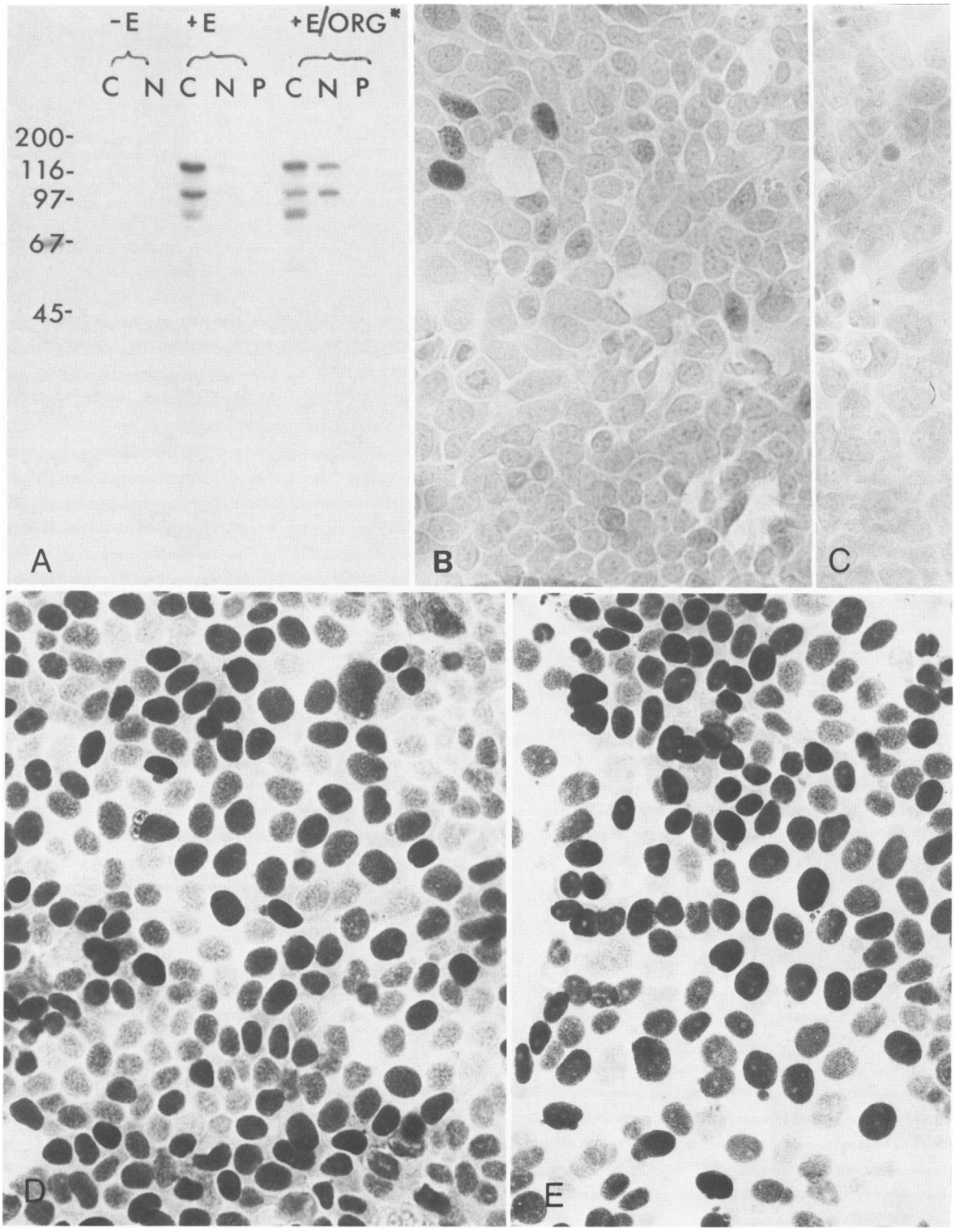

Figure 2. *Identification of PR with monoclonal PR antibody.* **A:** *Immunoblot analysis of cytosolic extract (C), nuclear extract (N), and residual pellet (P) from MCF-7 cells that received no estradiol (−E), were treated with long-term estradiol (5 nM, 4 days) but no progestin (+E), or were treated with long-term estradiol (5 nM, 4 days) followed by short-term progestin (+E/ORG*). Proteins of approximately 120 kd and 95 kd, corresponding to the A and B components of PR, were identified after 4 days of estrogen treatment. In addition, another band of approximately 87 kd was identified. Based on previous experience with these antibodies,*[18] this band was considered to be a break-down product of PR protein. **B–E:** *Immunohistochemical localization of PR in MCF-7 cells. Without estradiol treatment, PR can be identified in only a few, isolated cells* **(B)**. *Exclusively nuclear localization of PR is obtained either without* **(D)** *or with* **(E)** *short-term progestin treatment after PR induction by long-term estradiol administration. The intensity of nuclear staining in both* **D** *and* **E** *varied in individual cells, but most cells showed intense or strong immunostaining with very few nonstained nuclei. Negative controls* **(C)** *treated with normal rat IgG instead of rat monoclonal PR antibody show only nonspecific staining (no counterstain, ×400).*

ing fixatives followed by multiple washes.[32] This indicates that our methods of tissue fixation and processing should be sufficient to permit immobilization of extranuclear receptor, especially if it were present in the quantities indicated by the cytosolic extracts.

Although steroid binding assays and immunoblot analyses provided data consistent with the concept that an unoccupied cytoplasmic receptor is "translocated" to the nucleus after binding its ligand, immunocytochemistry showed an exclusively nuclear distribution of receptor both in the presence and absence of ligand. Why was the distribution of ER and PR with the immunohistochemical assay different from the results with both the biochemical and immunoblot analyses? Our current interpretation of these results is that the unoccupied forms of ER and PR, although nuclear proteins, are not tightly bound to nuclear components and are easily extracted from nuclei during the hypotonic lysis of the cells required for both conventional steroid-binding assay and immunoblot analysis.[4,5] The occupied forms of ER and PR, on the other hand, are transcriptional activators interacting with nuclear components[34] in some way that makes them more resistent to extraction from nuclei. Immunocytochemical assay does not require disruption of the cells and separation of the cellular components. Receptor proteins, therefore, are unlikely to be anatomically redistributed. The observation that ER and PR are nuclear proteins despite their appearance in cytosolic extracts strongly supports previous reports.[1-5] At least two other members of the family of steroid hormone receptor gene products, the androgen receptor[35,36] and the vitamin D_3 receptor,[37] are also nuclear proteins.

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