Journal of Chromatography, 508 (1990) 97–107
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 271

One-step 10^4-fold purification of transformed glucocorticoid receptor

Method for purifying receptors associated with M_r *ca.* 90 000 heat-shock protein

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(First received October 3rd, 1989; revised manuscript received January 10th, 1990)

SUMMARY

Chromatography of rabbit glucocorticoid–receptor complexes in the absence of sodium molybdate on a Mono Q anion-exchange column induces the transformation of the receptor and allows the resolution of the transformed and non-transformed molecular species. These abilities were used to design a new purification scheme for the glucocorticoid receptor from rabbit liver in its transformed state. Microgram amounts of receptor were obtained using this single-step procedure in less than 2 h. The purification yield was 50–60%. Immunoblot experiments showed that the glucocorticoid receptor was present as an $M_r \approx 94\,000$ polypeptide in these preparations and represented 20–30% of the eluted proteins as determined by densitometric scanning analysis of silver-stained sodium dodecyl sulphate polyacrylamide gels. Finally, the purified receptor was able to interact quantitatively with bulk DNA.

INTRODUCTION

Glucocorticoid hormone receptors are soluble proteins, present at low concentration in target cells (0.01–0.1‰ of total cytosolic proteins). Two main procedures for glucocorticoid receptor purification have been used. Ligand affinity chromatography is based on the ability of the receptor to bind specifically its ligand (for a review, see ref. 1). The second technique is based on the capacity of the glucocorticoid receptor to be converted into a DNA-binding form following ligand binding and heat treatment[2]. Sequential chromatography on phosphocellulose and DNA–cellulose has been used to purify glucocorticoid–receptor complexes from rat liver[3,4], porcine liver[5] and human HeLa S3 cells[6]. The purified receptor preparations thus obtained have been very useful, *e.g.*, in raising polyclonal[7] and monoclonal antibodies[8], defining the

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domain structure of the glucocorticoid receptor protein[9] and studying the interaction of the receptor with specific DNA sequences[10–13].

The characterization and purification of glucocorticoid–receptor complexes have mainly been performed using conventional low-pressure chromatographic techniques. More recently, high-performance liquid chromatographic methods have been shown to have interesting preparative[14,15] and analytical[16–19] applications. We report here on the use of high-performance ion-exchange chromatography (HPIEC) to purify rapidly and efficiently the transformed glucocorticoid receptor. This new method relied on two aspects: (i) the high resolution of HPIEC, allowing transformed and non-transformed glucocorticoid–receptor complexes to be separated and (ii) the matrix-induced transformation of the immobilized receptor. Characterization of the chromatographic eluates indicated that the purified receptor was a molecular mass, $M_r \approx 94\,000$ protein which was recognized by a monoclonal antibody and was able to interact with DNA.

EXPERIMENTAL

Materials

[1, 2, 4(n)-^{3}H]Dexamethasone (45 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, U.K.), unlabelled dexamethasone from Roussel UCLAF (Romainville, France) and DEAE-Trisacryl M, ss DNA-Ultrogel A4R and HA-Ultrogel from IBF (Villeneuve la Garenne, France). All other chemicals were of analytical-reagent grade from Sigma (St. Louis, MO, U.S.A.).

Preparation of cytosol

Cytosol from adrenalectomized rabbits was prepared in PG buffer [20 m*M* potassium phosphate, 20% (v/v) glycerol, 20 m*M* 2-mercaptoethanol, pH 7.4] or in PGM buffer (10 m*M* sodium molybdate in PG buffer) as described[20]. Receptor was labelled with 20 n*M* [^{3}H]dexamethasone for 16 h at 4°C. Non-specific binding was measured by a parallel incubation with a 1000-fold excess of unlabelled dexamethasone and steroid-binding activity was determined according to Blanchardie *et al.*[20]. The starting cytosol contained *ca.* 3–4 pmol/ml of [^{3}H]dexamethasone-binding sites and 15 mg/ml of proteins.

High-performance ion-exchange chromatography

HPIEC was performed using a Beckman chromatographic system equipped with a Model 420 gradient programmer controlling two Model 110A pumps. Samples were chromatographed on a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). The flow-rate and the composition of the mobile phase were controlled by the gradient programmer. The buffers used were (A) 20 m*M* Tris–HCl–20 m*M* 2-mercaptoethanol–2% (v/v) acetonitrile–0.2 m*M* phenylmethylsulphonyl fluoride (PMSF) (pH 7.4) and (B) 0.5 *M* sodium chloride–10 m*M* sodium molybdate in buffer A. Acetonitrile appeared to accelerate significantly the removal of free dexamethasone during the washing steps without affecting the ligand binding to the receptor (data not shown). Further, it was helpful to dissolve the PMSF, used as a protease inhibitor during the purification procedure. All samples and buffers were filtered through a 0.45-μm filter before use. The programme used to purify the transformed glucocorticoid receptor from rabbit liver is detailed in Table I.

TABLE I

PURIFICATION OF RABBIT LIVER GLUCOCORTICOID RECEPTOR

Step	*Time (min)*	*Buffer B (%)*	*NaCl (mM)*	*Na_2MoO_4 (mM)*
Sample + wash	0→20	50	250	5
Transformation	20→65	0	0	0
Elution	65→90	0→100	0→500	0→10

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared and electrophoresed under denaturing conditions according to Laemmli[21] as described in detail elsewhere[22]. After electrophoresis, the gels were silver stained[23] and scanned on an LKB Ultroscan XL. Calibration was performed using the following prestained standard proteins: myosin ($M_r \approx 200\,000$), phosphorylase *b* ($M_r \approx 92\,000$), bovine serum albumin ($M_r \approx 69\,000$) and carbonic anhydrase ($M_r \approx 30\,000$).

Protein immunoblot

Proteins separated by SDS-PAGE were transferred to nitrocellulose filters[22]. Glucocorticoid receptor immunoreactivity was detected using the monoclonal antibody No. 7 raised against the transformed rat liver glucocorticoid receptor[8] followed by reaction with goat anti-mouse immunoglobins labelled with horseradish peroxidase[22].

Separation of glucocorticoid–receptor complexes

The DNA-binding activity of purified glucocorticoid–receptor complexes was determined using a modification[22] of the procedure described by Holbrook *et al.*[24]. Briefly, three syringes containing 0.2 ml of DNA-Ultrogel (on the top), 0.2 ml of DEAE-Trisacryl (in the middle) and 0.2 ml of HA-Ultrogel (on the bottom) were connected to one other. Samples to be analysed were diluted 5-fold with PGM buffer and loaded on the minicolumns. The gels were then washed with PGM buffer (*ca.* 20 ml) and counted. The results are expressed as the percentage of radioactivity retained on each gel.

Miscellaneous

Quantification of proteins was performed according to the method of Bradford[25] using bovine serum albumin as a standard. Radioactivity was measured in a Beckman LS 2800 liquid scintillation counter. The concentration of chloride ions was determined using an Astra analyser (Beckman).

RESULTS

Binding capacity of receptor to the Mono Q column

We have previously shown that the non-transformed glucocorticoid receptor from rabbit liver, stabilized by sodium molybdate, is retained on a Mono Q anion-

exchange column[18]. In order to determine the capacity of this matrix, we incubated batchwise increasing amounts of labelled liver cytosol (10 μl–1 ml) with 10-mg aliquots of stationary phase. Following incubation, unbound proteins and free ligand were washed away, and the retained glucocorticoid–receptor complexes were eluted with 0.5 *M* sodium chloride solution. The saturation curve obtained after determination of the radioactivity eluted from the aliquots of gel is shown in Fig. 1A. These results were also analysed according to Scatchard[26] as shown in Fig. 1B. The number of binding sites for glucocorticoid–receptor complexes on the matrix was calculated to be 0.78 pmol per 10 mg of stationary phase. Similar results were obtained for anion-exchange matrices used in conventional chromatography (data not shown). Rabbit liver cytosol usually contains 0.2–0.3 pmol of glucocorticoid binding sites per milligram of protein[20]. We were then able to calculate that glucocorticoid–receptor complexes from 8–10 ml of cytosol could be retained on a 1-ml column (Mono Q HR 5/5).

Induction of transformation by the anion exchanger

Chromatography on a Mono Q column allows the complete resolution of transformed complexes generated by heat or salt treatment and non-transformed complexes[18]. When labelled cytosol was chromatographed in the absence of molybdate, a less negatively charged dexamethasone-binding species was eluted by a linear salt gra-

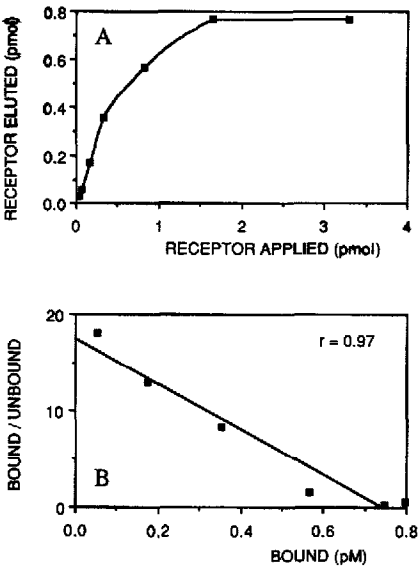

Fig. 1. (A) Binding of [^{3}H]dexamethasone–receptor complexes to the anion exchanger. Aliquots of cytosol (10 μl–1 ml), labelled with [^{3}H]dexamethasone in the absence or presence of a 1000-fold excess of unlabelled dexamethasone, were incubated for 15 min at 4°C with 10 mg of solid phase from a Mono Q column. The solid phase was recovered by centrifugation, washed with 1 ml of buffer A and retained glucocorticoid–receptor complexes were eluted with 0.25 ml of buffer B. The amount of specific radioactivity contained in these eluates was determined by scintillation counting and the number of receptors eluted was plotted against the amount of receptor applied on the gel. (B) Scatchard representation of the experimental results.

dient. This new receptor form, which was not detected when sodium molybdate was included in all the buffers, has been identified as the transformed receptor ($M_r \approx$ 100 000 DNA-binding species[18]).

We then tested the effect of different washing conditions on the yield of the transformation of glucocorticoid–receptor complexes adsorbed on the stationary phase. Labelled cytosol was loaded on the Mono Q column, which was washed with molybdate-free buffer at 4 or 20°C. The [^{3}H]dexamethasone–receptor complexes were then eluted with a linear salt gradient and the two peaks were pooled separately and assayed for radioactivity. The results obtained are shown in Fig. 2, where the percentages of transformation correspond to the ratio of the radioactivity in the peak of transformed receptor to that in the two peaks. In all instances, the percentage of transformation (*i.e.*, the relative abundance of the more acidic species) increased with the length of the washing step (Fig. 2). Half of the steroid–receptor complexes prepared in molybdate-containing buffer were in the transformed state after a 15-min washing period with buffer A at 4°C (Fig. 2, closed squares). Heating the column at 20°C during the washing period considerably accelerated this phenomenon as 70% of the complexes prepared in the presence of molybdate were transformed after only 5 min (Fig. 2, open squares). The same extent of transformation was obtained after 15–20 min when the cytosol applied to the column was prepared without sodium molybdate and washed at 4°C (Fig. 2, closed circles).

Purification of glucocorticoid receptor

While the proteins that were eluted from the Mono Q column below *ca.* 0.25 *M* sodium chloride in the presence of molybdate were washed away during the first step of the purification protocol, the molybdate-stabilized non-transformed glucocorticoid–receptor complexes, normally eluted with *ca.* 0.32 *M* sodium chloride[18], were still retained on the column. Interaction of these complexes with the cationic matrix in the absence of molybdate was shown to induce their transformation, even when the cytosol was prepared in molybdate-containing buffer (Fig. 2). Therefore, the receptors were converted to the more acidic species during the second phase of the purification, *i.e.*, when the column was washed with a mobile phase containing no salt and

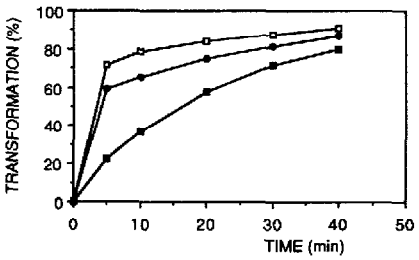

Fig. 2. Transformation of glucocorticoid–receptor complexes induced by the anion exchanger. Cytosol prepared in (●) PG buffer or in (□, ■) PGM buffer was incubated with 20 n*M* [^{3}H]dexamethasone for 16 h. Samples (500 μl) were loaded on a Mono Q column. After washing with buffer A for different times at (●, ■) 4°C or (□) 20°C, elution was carried out with a linear salt gradient (buffer A to buffer B in 25 min). The amount of radioactivity contained in each peak was determined, and the ratio of radioactivity in the peak of transformed receptor (first peak eluted) to total radioactivity eluted was calculated and plotted against the length of the washing step.

no molybdate. The retained proteins were finally eluted with a linear salt gradient (Fig. 3B). An aliquot of each fraction was counted in order to localize glucocorticoid–receptor complexes (Fig. 3A, closed squares). Comparison with the elution profile of proteins (Fig. 3A, open diamonds) indicated that the protein concentration in the fractions containing the transformed receptors was very low. This was not surprising as proteins which eluted below 0.25 *M* sodium chloride have been eliminated during the first phase of the purification, and the final eluate contained exclusively proteins for which the total negative charge had been greatly decreased by the elimination of sodium molybdate during the washing step. It is interesting that the major peak of protein, eluted at 0.3–0.4 *M* sodium chloride, contained large amounts of partially purified $M_r \approx 90\,000$ heat-shock protein, hsp90[22].

Characterization of the purified receptor

The fractions corresponding to the first peak of eluted radioactivity were pooled and the glucocorticoid receptor present in these preparations was characterized by several criteria. The proteins contained in these fractions were precipitated with trichloroacetic acid, dissolved in solubilization buffer and analysed by denaturing PAGE. Following transfer to sheets of nitrocellulose, glucocorticoid receptor was probed using the monoclonal antibody No. 7 raised against the rat receptor[8]. A major band of immunoreactivity was detected on the filter corresponding to an $M_r \approx$ 94 000 protein (Fig. 4, lane 1). This value is in complete agreement with the reported molecular weight determined under similar conditions for rat[4], porcine[5] and human glucocorticoid receptor[6].

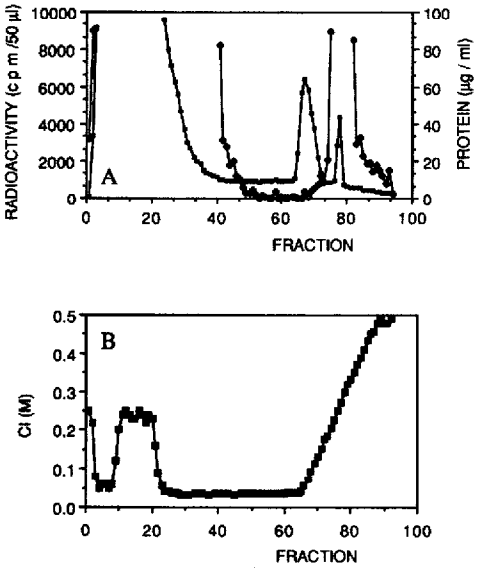

Fig. 3. Chromatographic profile of a typical purification. Cytosol from rabbit liver was incubated with 20 n*M* [^{3}H]dexamethasone for 16 h at 4°C and 8 ml were loaded on a Mono Q column washed with 50% of buffer B for 20 min. The column was then washed with buffer A for 45 min and the elution was initiated. Each fraction collected was assayed for (A, ◇) protein content (A, ■) radioactivity and (B) chloride ion concentration.

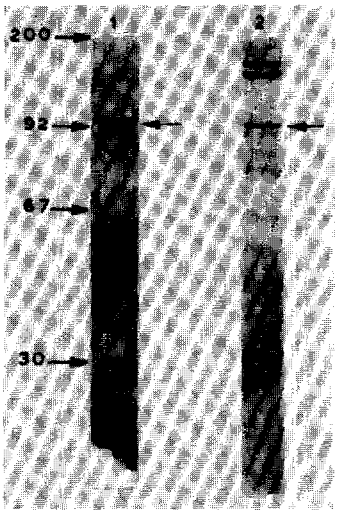

Fig. 4. SDS-PAGE of purified receptor. Samples containing purified receptor (*ca.* 0.5 μg) were analysed under denaturing conditions. Lane 1 shows an immunoblot analysis of the eluate probed with the monoclonal anti-glucocorticoid receptor antibody N. 7. A similar gel was silver stained (lane 2). Positions of standard proteins are shown on the left (molecular weights × 10^{-3}). The arrows on the right indicate the position of the glucocorticoid receptor.

When the polyacrylamide gel was silver stained, a band was clearly seen in the region where the immunoreactivity was detected (Fig. 4, lane 2). Densitometric scanning analysis of such gels revealed that the $M_r \approx 94\,000$ receptor protein represented 20–30% of the eluted proteins (data not shown). Using this technique to determine the purity of the preparation, and assuming one hormone-binding site per receptor molecule, it was possible to calculate that the glucocorticoid–receptor complexes were purified *ca.* 10 000-fold by this single step (Table II). Two other proteins ($M_r \approx 140\,000$ and $M_r \approx 155\,000$) were seen on silver-stained gels. At present, there has been no report indicating the presence of such high-molecular-weight proteins in the non-transformed glucocorticoid receptor. It is possible, however, that these proteins

TABLE II

PURIFICATION OF RABBIT GLUCOCORTICOID RECEPTOR

The binding activity of the cytosol was determined as described under Experimental. The purification was performed using 8 ml of labelled cytosol as starting material.

Step	*Proteins (mg)*	*Receptor (pmol)*	*Purity (%)*	*Purification (fold)*	*Yield (%)*
Cytosol	146	38.5	0.0025[a]	1	100
Mono Q Eluate	–	21.8	28.1[b]	11 336	56.5

[a] Calculated assuming a molecular weight of 94 000.

[b] Determined by densitometric scanning analysis of SDS-PAGE after silver staining.

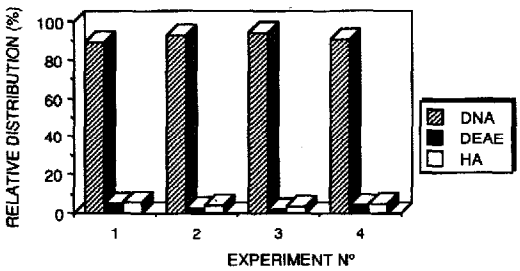

Fig. 5. DNA-binding ability of purified glucocorticoid–receptor complexes. Purified samples eluted from the Mono Q column were diluted with PGM buffer and chromatographed on minicolumns as detailed under Experimental. The retentions on (DNA) DNA-Ultrogel, (DEAE) DEAE-Trisacryl and (HA) HA-Ultrogel of purified complexes from four separate purifications are presented.

also associate with hsp90 to form complexes that are stabilized by sodium molybdate. These complexes might have been dissociated when the Mono Q column was washed with molybdate-free buffer, releasing the monomeric $M_r \approx 140\,000$ and $M_r \approx 155\,000$ proteins.

Characterization of the purified receptor was also performed under non-denaturing conditions. The hydrodynamic parameters determined were similar to those previously reported for the transformed receptor from rabbit liver[18], *i.e.*, a Stokes radius of 5.0–5.2 nm, a sedimentation coefficient of *ca.* 4.5 S and a calculated molecular weight of *ca.* 100 000 (data not shown). Hence, the receptor purified by this procedure appeared to be in a monomeric form, as opposed to the dimeric state recently reported by Wrange *et al.*[27].

The property of DNA binding was studied by the minicolumn procedure originally described by Holbrook *et al.*[24]. The results from four separate experiments are presented in Fig. 5. It is clear from this diagram that most of the purified receptors are present in a DNA-binding form, as expected for a receptor in a transformed state.

Conservation of the purified receptor

The stability of the purified receptor preparations was tested. The eluates were supplemented with 10% (v/v) glycerol, 0.1% (w/v) bacitracin and 10 n*M* [^{3}H]dexamethasone and stored at 4°C or frozen at −70°C. At different times, the number of

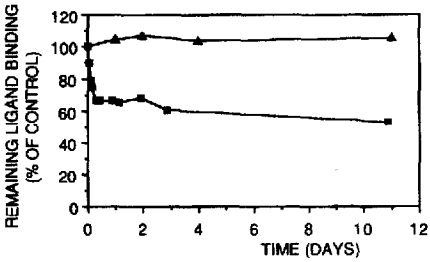

Fig. 6. Ligand-binding stability of the purified receptor. Purified samples were supplemented with 10% glycerol, 0.1% (w/v) bacitracin and 10 n*M* [^{3}H]dexamethasone. Aliquots were assayed for the presence of glucocorticoid–receptor complexes after different times of storage at (■) 4°C or (▲) −70°C.

ligand-binding sites remaining in the samples was determined using the hydroxylapatite assay[28]. The non-specific binding was measured after exposure of the eluates to 100°C for 10 min. At 4°C, the ligand-binding capacity of the purified receptors was decreased to *ca.* 60% after 4 h, but then remained stable for as long as 11 days (Fig. 6). When the samples were stored at −70°C, no decrease in the ligand-binding capacity was seen (Fig. 6), and the purified receptor was still able to interact with DNA (data not shown).

DISCUSSION

Transformation of the glucocorticoid receptor can be induced *in vitro* by a variety of manipulations such as heat treatment, exposure to elevated ionic strength, dilution, ATP or heparin (for a review, see ref. 29). The appearance of DNA-binding activity appears to correlate with the dissociation of the dimer of hsp90 from the M_r 94 000 receptor monomer[30–32]. This transformation also occurs when the receptor is immobilized on an immunoaffinity matrix[2,32] or on an anion exchanger[15,18,33]. The nature of this phenomenon still remains to be elucidated. However, it seems to be well established that most of the hydrodynamic and physico-chemical properties of the non-transformed glucocorticoid receptor are related to the presence of a dimer of hsp90 in the complex. This protein is highly negatively charged[34,35], which is probably responsible for the behaviour of the molybdate-stabilized non-transformed glucocorticoid receptor on anion exchangers[36–38]. When the receptor is adsorbed on the Mono Q column in the absence of molybdate, the hsp90 dissociates and the total negative charge of the glucocorticoid–receptor complex dramatically decreases.

We combined the high resolution of HPIEC with this transformation process to develop a new purification scheme. This protocol, as opposed to the purification techniques routinely used (see Introduction and the references cited there), does not rely on the properties of ligand and DNA binding of the receptor. The purified receptor appeared to be intact as judged by its molecular weight determined under denaturing conditions. It also contained the three functional domains first described at the protein level by Carlstedt-Duke *et al.*[39]. Thus, the C-terminal third of the purified receptor was bound to the ligand, the DNA-binding domain was accessible since the receptor could be retained on immobilized DNA and the purified protein was recognized by a monoclonal antibody raised against the N-terminal "immunodominant" domain[39]. The function of the glucocorticoid receptor is to regulate the transcription of specific genes[40]. However, there has so far been no report describing an *in vitro* transcription system that we could use to determine whether the glucocorticoid receptor purified according to our procedure is functional or not. Finally, the purified material was stable, especially at −70°C, and the preparations could be stored until further use.

The different sequences of the purification (washing with 0.25 *M* sodium chloride and 5 m*M* sodium molybdate, washing with no salt and no molybdate, salt gradient) were automatically controlled by the gradient programmer. No manual operation was required after injection of the cytosolic preparation. The purified receptor was eluted only *ca.* 70 min after the injection, and a new purification could be performed every 90 min. Starting with 8–10 ml of rabbit liver cytosol, we routinely obtained microgram amounts of purified receptor. Similar results were obtained us-

ing triamcinolone acetonide-labelled rat liver glucocorticoid receptor as starting material[41]. As 5- and 10-ml Mono Q columns are available larger amount of purified receptor can be obtained using this protocol.

We also tried to extend this purification technique to other steroid hormone receptors that are also found associated with the $M_r \approx 90\,000$ heat-shock protein in cytosol[42]. Preliminary experiments indicated that progesterone receptors from human T47D cells and estrogen receptors from human MCF7 cells are also transformed by interaction with the cationic matrix[43], and therefore can be purified using a similar scheme. The purification protocol described here and its straightforward application to other steroid hormone receptors would be of great convenience for experiments requiring microgram amounts of potentially functional receptor.

ACKNOWLEDGEMENTS

This work was supported by grants from INSERM, from the Association pour la Recherche contre le Cancer (ARC) and by U.F.R. of Medicine. We thank A.-C. Wikström and J.-Å. Gustafsson for the gift of monoclonal antibodies.

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