Journal of Cellular Biochemistry 79:471–485 (2000)

# Changes in the Expression of Cytokeratins and Nuclear Matrix Proteins are Correlated With the Level of Differentiation in Human Prostate Cancer

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**Abstract** The nuclear matrix-intermediate filament complex (NM-IF) is a protein scaffold which spans the whole cell, and several lines of evidence suggest that this structural frame represents also a functional unit, which could be involved in the epigenetic control of cancer development. Here we report the characterization by high resolution two-dimensional gel electrophoresis and Western blot analysis of the NM-IF complex isolated from prostate cancer (PCa); tumor-associated proteins were identified by comparing the electrophoretic patterns with those of normal human prostate (NHP). Extensive changes in the expression of both the NM and IF proteins occur; they are, however, related in a different way to tumor progression. Poorly differentiated PCa (Gleason score 8–9) shows a strong down regulation of several constitutive cytokeratins (CKs 8, 18, and 19); their expression significantly ($P < 0.05$) decreases with respect to both NHP and benign prostatic hyperplasia (BPH) and, more interestingly, also with respect to moderately (Gleason score 6–7) and well (Gleason score 4–5) differentiated tumors. Moreover, we have identified a tumor-associated species which is present in all of the tumors examined, systematically absent in NHP and occurs only in a few samples of BPH; this polypeptide, of $M_r$ 48,000 and pI 6.0, represent a proteolytic fragment of CK8. At variance with these continuing alterations in the expression, the NM proteins undergo stepwise changes correlating with the level of differentiation. The development of less differentiated tumors is characterized by the appearance of several new proteins and by the decrease in the expression of others. Six proteins were found to be expressed with a frequency equal to one in poorly differentiated tumor, namely in all the samples of tumor examined, while in moderately and well differentiated tumors the frequency is less than one, and decreases with increasing the level of differentiation. When tumors of increasing Gleason score are compared with NHP a dramatic increase in the complexity of the protein patterns is observed, indicating that tumor dedifferentiation results in a considerable increase in the phenotypic diversity. These results suggest that tumor progression can be characterized using an appropriate subset of tumor-associated NM proteins. J. Cell. Biochem. 79:471–485, 2000. 

**Key words:** prostate carcinoma; nuclear scaffold; intermediate filaments; tumor-associated proteins; chromatin structure

Recent advances in the understanding of mechanistic aspects of cancer development as well as in the identification of new diagnostic and prognostic markers rely on the analysis of the protein changes the nuclear matrix (NM) undergoes during tumor progression [Pienta et al., 1989; Keesee et al., 1996; Nickerson, 1998]. Following the identification in human prostate cancer (PCa) of a tumor specific NM protein [Partin et al., 1993] other studies have extended this approach to breast [Khanuja et al., 1993], colon [Keesee et al., 1994], bladder [Getzenberg et al., 1996], head, and neck [Donat et al., 1996]. In every case, cancer development has been found to be associated with the expression of several new proteins, and with the cessation of the expression of others. Other workers focused on a more inclusive structural frame, the so-called NM-intermediate filament

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Received 18 January 2000; Accepted 17 May 2000

(IF) complex [Barboro et al., 1996; Coutts et al., 1996; Prasad et al., 1998]. Different lines of evidence suggest, indeed, that the NM-IF complex represents a functional unit, involved in signal transduction from the cell periphery to the DNA [Pienta and Coffey, 1992]. First, the interaction of both normal and transformed rat kidney cells with an extracellular matrix secreted by tumor cells induces modifications in the protein compositions of both the IFs and the NM [Getzenberg et al., 1991]. Second, it has been suggested that the ability of some tumors to co-express vimentin and cytokeratins (CKs) confers to the cells a selective advantage in the response to signals from the extracellular matrix [Hendrix et al., 1996]. Moreover, the architecture of the NM-IF complex is dramatically altered by tumor promoters [Fey and Penman, 1984].

In an exploratory investigation on the role of the structural changes of chromatin in cancer development using different biochemical and biophysical methods [Barboro et al., 1993], we noted that in the resistant hepatocyte model of Solt and Farber the heterochromatin content progressively decreases during the evolution of hepatocyte nodules, suggesting that the process of transformation involves a large increase in the fraction of chromatin existing in a state of transcriptional competence. Comparable observations on the occurrence of extensive rearrangements and unfolding of chromatin in transformation have also been presented by other groups [Leonardson and Levy, 1989; Laitinen et al., 1990]. To start the identification of general modulating factors of chromatin superstructure we have recently carried out the protein characterization of the NM-IF complex during rat hepatocyte transformation; we have found that the protein composition undergoes progressive changes synchronous with chromatin decondensation [Barboro et al., 1996]. The new synthesis of NM proteins relating to the emergence of successive cell populations was observed, in line with the contention that a subset of NM proteins is characteristic of the stage of transformation [Pienta et al., 1989; Brancolini and Schneider, 1991]. The actual relationship between chromatin decondensation phenomena and alterations in the composition and/or architecture of the NM still remains to be discovered. We have recently shown, however, that when chromatin is released from the constraints arising from the anchoring of the loop to the NM a catastrophic (all-or-none) decondensation process occurs [Balbi et al., 1999], showing that changes in the interaction between DNA or chromatin with proteins of the nuclear scaffold can directly trigger major structural rearrangements in the bulk of the chromatin. Moreover, transformed hepatocytes were found to express an inappropriate CK with $M_r$ of 39,000 analogous to human CK19, its expression steadily increases during the evolution of hepatocyte nodules, suggesting that this species might be involved in the epigenetic control of transformation through the rearrangement of the cytoskeleton [Barboro et al., 1996].

The reason why in this work we have extended the characterization of the NM-IF complex to PCa is two-fold. In the first place we aimed at verifying whether or not the sequential changes in the protein patterns of the NM-IF complex which occur in an experimental model of chemical carcinogenesis can also be observed in the course of the evolution of a human tumor. Moreover, PCa is the most common malignant tumor in men and in Western countries is the second cause of male cancer mortality [Satariano et al., 1998]. In spite of this, the molecular events involved in tumor progression are poorly understood both at the genetic and epigenetic level [Karp et al., 1996; Lalani et al., 1997]. Differences in the expression of CKs between normal, hyperplastic, and tumor cells from human prostate have been documented early by immunoblot analysis by Sherwood et al. [1990]. Partin et al. [1993] have reported for the first time the presence of a specific NM protein (PC-1) in prostate cancer; very recently work from the same laboratory [Lakshmanan et al., 1998] has described another PCa specific protein (YL-1), whose expression appears to be correlated with the pathological stage.

In a recent report, we have presented the results of a preliminary characterization of the NM-IF complex isolated from PCa [Alberti et al., 1996]. Both NM proteins and IFs showed qualitative and quantitative changes in PCa compared to benign prostatic hyperplasia (BPH). This paper reports the identification of several tumor-associated NM proteins, and furthermore shows that the expression of the major CKs (8, 18, and 19) undergoes a sharp decrease in poorly differentiated PCa.

**TABLE I. Patient Characteristics**

| Number of patients | Median age, year (range) | Diagnosis |
|---|---|---|
| 5 | 63 (52–76) | NHP |
| 17 | 64 (51–71) | BPH |
| 6 | 67 (59–69) | PCa (Gleason 4–5) |
| 14 | 64 (57–74) | PCa (Gleason 6–7) |
| 9 | 60 (51–71) | PCa (Gleason 8–9) |

## MATERIALS AND METHODS

### Tissue Samples

Neoplastic tissue specimens were obtained from 29 patients undergoing radical retropubic prostatectomy for PCa. BPH was obtained from the prostatic lobe controlateral to the cancer zone. NHP samples were collected from five patients undergoing cistectomy for bladder cancer. Samples, not exceeding a diameter of 1 cm, were immediately frozen in liquid nitrogen. All tissues were histologically confirmed by hematoxylin and eosin staining of frozen sections adjacent to those used to isolate the NM-IF complex. The patient characteristics are summarized in Table I; the tumors were classified according to the TNM system and graded according to the criteria of Gleason [Rosai et al., 1993].

### Isolation of the NM-IF Complex

The NM-IF complex was isolated according to Barboro et al. [1996] with minor modifications. In order to minimize both proteolysis and artifactual cross-linking by disulfide bridges of IF proteins during the preparation, all the buffers were supplemented with 5 mM $Na_2S_2O_5$, 1 mM phenylmethylsulphonilfluoride, 0.5 mM benzamidine, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 25 μg/ml aprotinin, and 1 mM dithiothreitol; all the steps were carried out at 4°C. From 200–400 mg of tissue specimen were minced into 1 $mm^3$ pieces, homogenized in 15 ml of 75 mM NaCl and 24 mM $Na_2EDTA$, pH 7.8, using a potter with a Teflon pestle, and sieved through a 230 mesh stainless steel grid to trap the fibrous elements of the tissue; this procedure removes stromal aggregates, and yields an almost homogeneous preparation of epithelial cells. The cells were harvested by centrifugation at 150*g* for 15 min. The pellet was resuspended in four volumes of 75 mM NaCl, 24 mM $Na_2EDTA$, 0.5% Triton X-100, pH 7.8. After a 5 min incubation the suspension was centrifuged at 150*g* for 5 min and washed three times in digestion buffer consisting of 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8, and 2 mM vanadyl adenosine to inhibit ribonuclease activity and digested for 1 h at 25°C with 400 μg/ml DNase I. Chromatin was removed by adding $(NH_4)_2SO_4$ from an 1 M stock solution to a final concentration of 0.25 M under gentle shacking. The NM-IF complex was recovered by centrifugation at 6,500*g* for 15 min, and extracted once again with a large excess of digestion buffer containing 0.25 M $(NH_4)_2SO_4$. The sample was again pelleted and solubilized for one- or two-dimensional gel electrophoresis as described below.

### Gel Electrophoresis

High-resolution two-dimensional gel electrophoresis was performed by isoelectric focusing followed by SDS-PAGE [O'Farrell, 1975]. The NM-IF complex was solubilized as described by Gerace et al. [1984]. About 10 mg of wet pellet, corresponding approximately to 0.2 mg of protein were resuspended in 20 μl of 1% SDS, 9.5 M urea, 1 mM $Na_2EDTA$, 50 mM dithiothreitol and 2% ampholine (composed of 8.5% pH range 4–6, 33% pH range 5–7 and 3.5–10, 25% pH range 5–8). After a 15 min incubation at 37°C, an equal volume of 10% Nonidet P-40, 2% ampholine and 9 M urea was added. In order to load equal protein amounts on the gels, the concentration of the solutions was determined before electrophoresis as described below. Sixty μg of NM-IF proteins (corresponding to from 13 to 15 μl of solution, depending on concentration) were then applied to 4% polyacrylamide tube gels 18 cm long and 1 mm in inner diameter containing 2% ampholine and separated at 300 V constant voltage for 76 h. After isoelectric focusing and equilibration for 3 min in a buffer containing 5% SDS, 50 mM dithiothreitol, 10% glycerol, 0.27 M Tris-HCl, pH 6.8, the tube gels were sealed to the top of a stacking gel which was on top of 8–15% gradient SDS-PAGE. Gel electrophoresis in the second dimension was carried out for 16 h at 5 mA/gel at a constant temperature of 12°C. Protein concentration was determined using the Bio-Rad protein microassay (Bio-Rad, München, Germany) with bovine serum albumin as a standard. One-microliter samples of protein solution in sample buffer [Gerace et al., 1984] were diluted with 0.8 ml of

water and 0.2 ml of dye reagent concentrate, and the concentration determined according to the manufacturer's instructions.

Relative molecular weights were determined using Pharmacia Electrophoresis Calibration Kits (Molecular weight 14,400–94,000; Pharmacia BioTech, Uppsala, Sweden); isoelectric points were determined using the carbamylated carbonic anhydrase standard (pI 4.8–6.7; Pharmacia BioTech). In addition, lamins A, B, and C, actin and vimentin were routinely used as internal standard. Proteins were detected by the silver staining procedure of Heukeshoven and Dernick [1988]. The detection limit of this highly sensitive method correspond to 0.05–0.1 ng of protein per band; in this work, however, we have considered significant only the spots containing about 1 ng of protein. By this criterion we selected only the spots having an optical density significantly higher than that of the background and, furthermore, the appearance of the spots is expected to be unaffected by slight changes in the developing conditions. The gels were scanned in a Kontron Elektronic Vidas 2.1 Image Analysis System (Kontron Elektronik, Eching, Germany) that produces grey level values that are linear with the specimen's trasmittance; calibration of the grey levels was carried out by submitting to two-dimensional gel electrophoresis known (from 0.1–20 ng) amounts of bovine serum albumin (Bio-Rad) and scanning the calibration gels. We have found that 1 ng of protein corresponds to a grey level ≈15% higher than that of the background, namely, to an optical density of ≈0.07.

One-dimensional SDS-PAGE was carried out according to Laemmli [1970] using 10% polyacrylamide gels; from 10–15 μg of proteins were loaded on the gels. Within this range the immunoreactive responses of all the major protein components, including the proteolytic fragments, were found to be strong enough to be carefully determined by densitometry (see below).

### Immunodetection of CKs

NM-IF proteins were resolved by one- or two-dimensional gel electrophoresis, and immunodetection of the CKs carried out as already described [Barboro et al., 1996] using the test antibodies reported in Table II. After incubation with the antibody the membranes were washed with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.1% Tween-20 (TBST) and incubated for 1.5 h with peroxidase-conjugated anti-mouse IgG and IgM (DAKO, Glostrup, Denmark) diluted to 1/1,000 in TBST containing 3% powdered nonfat milk, 100 U/ml penicillin G and 100 μg/ml streptomycin. Immunoreactive bands or spots were detected using an enhanced chemiluminescence assay kit (ECL, Amersham, Buckinghamshire, England). The same blot was used for three or four different antibodies. Before reprobing with a different antibody, the membranes were stripped by incubation with 2% SDS, 100 mM 2-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, for 30 min at 60°C, washed, tested for the absence of background, and blocked again.

**TABLE II. Specificity of the Antibodies Used in This Study**

| Antibody clone number | Specificity | Source | Dilution |
|---|---|---|---|
| 4.1.18 | CK8 | Boehringer | 1:400 |
| CK2 | CK18 | Boehringer | 1:400 |
| K4.62 | CK19 | Sigma | 1:300 |
| K8.12 | CK13, 15, 16 | Sigma | 1:300 |
| AE1/AE3 | CK 2, 4, 5, 8, 9, 10, 14, 15, 16, 19 | DAKO | 1:400 |
| V9 | Vimentin | Boehringer | 1:300 |

### Determination of the Relative Amounts of CKs in the NM-IF Complex

Ten μl samples of the same NM-IF preparation were loaded on two SDS 10% polyacrylamide gels and submitted to electrophoresis as described above. One gel was stained with Coomassie brillant blue R-250; as a rule five densitometric scans were performed in a Kontron Elektronic Vidas 2.1 image analysis system and the total amount of NM-IF proteins ($A$) evaluated by integration and subsequent averaging of the optical density curves; the determinations were quite accurate (the limits of the SE were ± 0.5%). After blotting the second gel was stained and the amount of residual proteins ($A_{res}$) determined as above. The efficiency of transfer of the proteins ($E$) was evaluated by the expression $(A–A_{res}/A)\times100$; from 10 experiments we obtained E = 87 ± 1.1% (mean ± SE), showing that under our experimental con-

ditions the protein transfer was well reproducible. The same procedure was used to establish whether different proteins were transferred with the same efficiency. No appreciable difference was detected in the $M_r$ range of interest (40,000–60,000).

Quantitation of the different CKs was obtained by densitometric analysis of the autoradiographies of the Western blots as described above, using Hyperfilm ECL films (Amersham), which exhibit a linear response to light produced from enhanced chemiluminescence. To verify further that the response was a linear function of the protein concentration, different amounts of the NM-IF complex (2–15 μg) were resolved on SDS-polyacrylamide gels. After immunoblotting, the integrated optical densities of the bands corresponding to different CKs were found to depend linearly on concentration in the range of interest. The relative amount of each CK was finally obtained by normalizing the integrated optical density obtained by ECL for the integrated optical density *A* of the corresponding Coomassie stained gel.

Date were analyzed with the SPSS for Window statistical package [Norusis, 1994]; the Kolnogorof-Smirnov (K-S) test was used to verify the normal distribution of the data, the one-factor ANOVA to analyze the variance. The statistical significance of the differences in the relative amounts of each CK among NHP, BPH and PCa samples was evaluated by the Tukey-B test. Differences was considered significant for $P < 0.05$.

## RESULTS

### Identification of the Major IF Proteins and Quantitation of the Differences in Composition Among NHP, BPH, and PCa

In a preliminary report [Alberti et al., 1996] we have shown that in PCa the composition of the XNM-IF complex undergoes both qualitative and quantitative changes with respect to BPH. In this study we have extended this comparison to 19 new tumor specimens and to the corresponding BPH; furthermore, we have characterized the NHP obtained from five patients undergoing cystectomy for bladder cancer. Individual IF components have been identified by two-dimensional immunoblot analysis using several monoclonal antibodies and quantified by densitometric scanning of one-dimensional immunoblots.

The results of the two-dimensional immunoblot analysis of the NM-IF isolated from NHP, using the monoclonal antibodies reported in Table II, are shown in Figure 1. Both CKs 8, 18, 19, and 15, which are constitutive of prostatic epithelia, and vimentin have been unambiguously localized in the gels. CK5, which represents the typical in vivo partner of CKs 14–15 cannot be focused in the first-dimension because its isoelectric point (7.4) is higher than pH 7.2, which corresponds to the basic end in our gels; it can, however, be easily detected in monodimensional gels (Fig. 2A). CKs 8 and 15 as well as vimentin have undergone partial proteolysis, as shown by the presence of fragments of lower molecular weight and more acidic isoelectric point, marked by arrowheads in Figure 1B, E, and F.

Quantitation of the differences in the expression of individual CKs has been obtained by monodimensional immunoblot analysis. A representative result is shown in Figure 2, where the immunoblot patterns of the CKs isolated from NHP, BPH, and PCas with different Gleason scores are compared: the tumors were grouped into low (Gleason score 4–5), intermediate (Gleason score 6–7), and high grade (Gleason score 8–9) lesions. The results of the densitometric analysis are reported in Figure 3. The relative amounts of CKs 8, 18, and 19 decrease significantly ($P < 0.05$) in poorly differentiated tumors (Gleason 8–9) with respect to both NHP and BPH and, more interestingly, also with respect to well and moderately differentiated tumors, while in the case of CK 5 and CKs 14–15 no statistically significant change has been found.

It might be objected that the decrease in the amounts of CKs 8, 18, and 19 merely reflects the changes in the histological pattern of PCa with respect to both NHP and BPH; in fact the tumor essentially comprises epithelial cells with little stroma elements which do not express CK polypeptides [Sherwood et al., 1989]. By routine hematoxylin and eosin analysis on frozen tissues 35 ± 5% (mean ± SE, five samples examined), 51 ± 6% (13 samples examined), and 79 ± 4% (15 samples examined) of the cells were found to be epithelium components in NHP, BPH, and PCa, respectively. The cellular populations obtained after sieving the tissue homogenates (see Materials and Methods) were characterized both for cellular morphology and by a standard immunocyto-

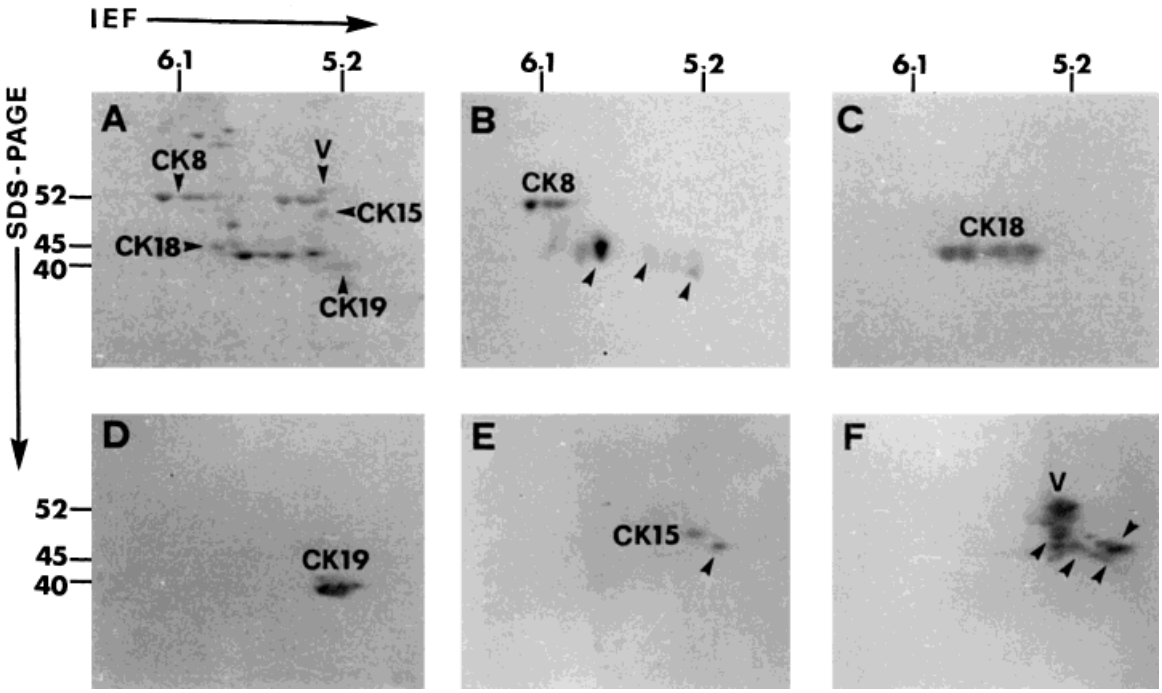


**Fig. 1.** Western blot analysis of the NM-IF complex isolated from NHP. Samples resolved by two-dimensional gel electrophoresis were either silver stained (**A**) or transferred to nitrocellulose membranes and probed with the monoclonal antibodies 4.1.18 (**B**), CK2 (**C**), K4.62 (**D**), K8.12 (**E**), and V9 (**F**). The specificity of the antibodies is reported in Table II. The major proteolytic fragments of CK8, CK15, and vimentin, V, are marked by arrowheads in B, E, and F, respectively.

chemical procedure, using the antibodies CAM 5.2, which is specific for CKs 8, 18, and 19 and AE1/AE3 in combination with immunoperoxidase staining; NHP, BPH, and PCa samples from the prostates of two, two, and three patients, respectively, were examined. All of the preparations were found to be almost homogeneous; from 95–100% of the cells were of epithelial nature independent of the primary tissue composition.

We have previously shown that the two tumor-associated proteins referred to as P8 and P5, which are systematically absent in NHP and only seldom observed in BPH, are recognized by the a-IFA [Alberti et al., 1996]; the molecular weight, isoelectric point, and occurrence in the different tissues are reported in Table III. In this work we have found that P8, of $M_r$ 48,000 and pI 6.0 (arrowhead in Fig. 4B), is stained by the antibody 4.1.18, which recognizes CK8 ($M_r$ 52,000 and pI 6.1) and represent therefore a proteolytic fragment of the latter. Since the isolation of the NM-IF complex has been carried out in the presence of high concentration of $Na_2EDTA$ and different inhibitors of proteases, we conclude that the degradation of CK8 pre-exists to the manipulation of the cells, and therefore represents an intrinsic feature of the malignant transformation.

It is well known that CKs are degraded by a specific $Ca^{2+}$-dependent protease, which remove positively charged peptides from one of the terminal segments of the molecule [Traub, 1985] giving rise to a characteristic staircase pattern of the fragments in two-dimensional gels. It is apparent that P8 represent the first product of the proteolysis of the more basic isoelectric variant of CK8 (marked by a circle in Fig. 4A) and therefore, its concentration will assume a stationary value independent on the extent of proteolysis, as a consequence of the stepwise mechanism of degradation. This is the reason why this fragment is present in small concentrations in all of the tumor examined, together with larger and variable amounts of low molecular weight proteolysis products (Fig. 4B). It has to be noted that proteolysis of CK8 is also observed in the NM-IF complex isolated from NHP (Fig. 4A); in this case, however, neither P8 nor other components of the staircase

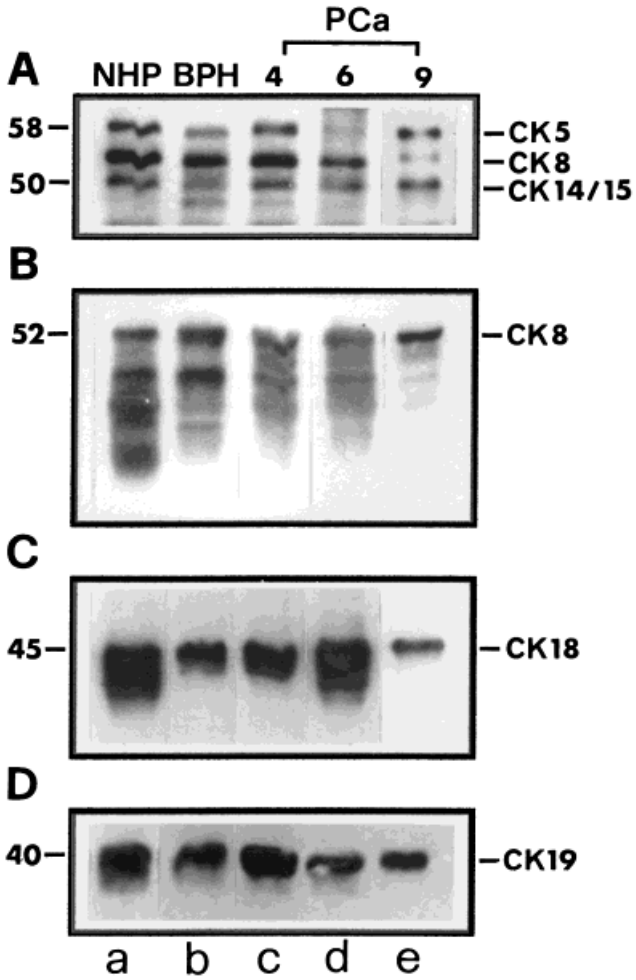


**Fig. 2.** Western blot analysis of the NM-IF complex isolated from NHP (lane a), BPH (lane b), and PCas with Gleason score 4, 6, and 9 (lanes c, d and e, respectively). Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with the monoclonal antibodies AE1/AE3 (**A**), 4.1.18 (**B**), CK2 (**C**), and K4.62 (**D**). The position of the molecular weight standards (in thousands) are indicated on the left.

pattern can be detected excepting a fragment of Mr 46,000 and pI 5.7. It is likely that the striking difference in the patterns of proteolytic fragments reflects tumor associated changes in the higher-order structure of IF proteins, which might result in the unmasking of the protease sensitive sites, rather than differences in the specificity of the degrading enzymatic activity. The former hypothesis has been recently put forward by Nishibori et al. [1996] in order to explain the accumulation of a tumor specific ubiquitin-conjugated CK8 fragment in human colorectal carcinomas.

As far as P5 is concerned, it did not significantly react with any of the antibodies reported in Table II, so that its immunological identification could not be further refined.

### Characterization of the Expression of Tumor-Associated NM Proteins

The emergence of the new cellular populations associated with tumor progression is characterized by the synthesis of several new NM proteins (Fig. 5). After careful calibration of the two-dimensional gels with pI and $M_r$ standards, several protein spots were unambiguously identified for each tissue sample. Excluding the area within which IF proteins and their fragments migrate, from 30 to 44 spots were detected in NHP, depending on the sample examined; from 41–67 in BPH and from 39–77, from 30–98 and from 64–140 in tumors of low, intermediate, and high Gleason score, respectively.

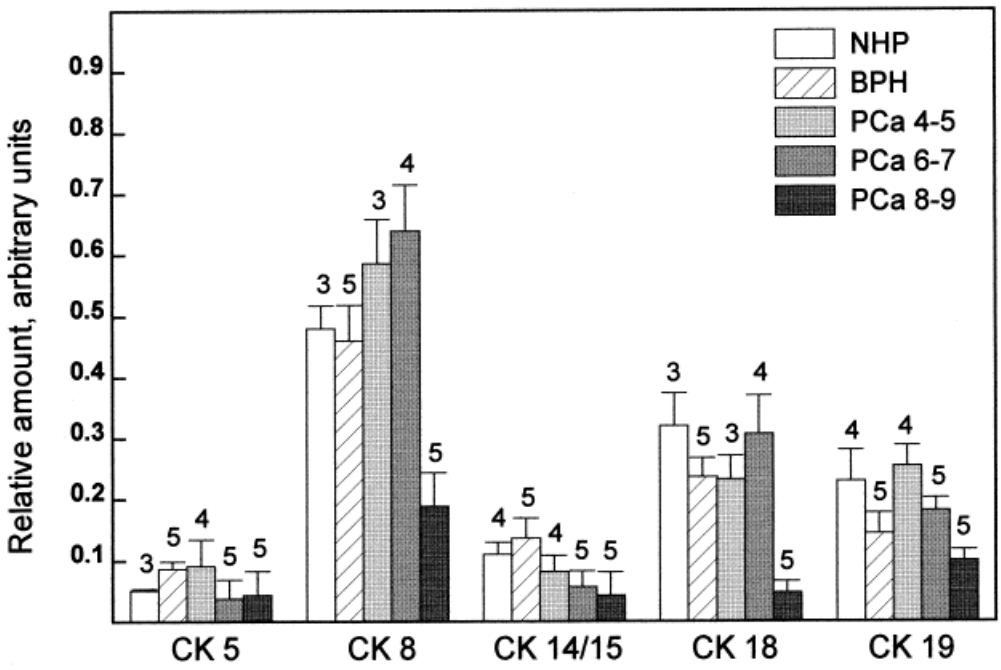


**Fig. 3.** Cytokeratin expression in NHP, BPH and PCas with different Gleason score. The ordinates represent the mean ± SE of the relative amounts of the different CKs determined by quantitative immunoblot analysis of from three to five tissue samples. The number of samples is reported above the corresponding rectangle; each sample was run at least on two gels. The relative amounts of CKs 8, 18, and 19 decrease significantly (Tukey-B test, $P < 0.05$) in poorly differentiated tumors (Gleason score 8–9) with respect to NHP, BPH, and PCas with Gleason score 4–5 and 6–7.

**TABLE III. Characterization and Relative Frequency of the Tumor-Associated IF Proteins P8 and P5 in NHP, BPH, and PCa**

| Protein | $M_r \times 10^{-3}$ | p.I. | NHP | BPH | PCa |
|---|---|---|---|---|---|
| P8 | 48 | 6.0 | 0 (0/5)[a] | 0.29 (5/17) | 1 (29/29) |
| P5 | 47 | 4.9 | 0 (0/5) | 0.29 (5/17) | 0.65 (19/29) |

[a]Number of positive samples/number of cases examined.

For the purpose of an immediate appreciation of the results, in Figure 6 the data are cast in the form of three–dimensional relative frequency diagrams. Each protein is identified by a pair of values in the IEF/SDS-PAGE plane, while the ordinate reports *F*, the relative frequency (number of positive samples/number of samples examined) with which the protein occurs in the tissue under consideration. Finally, the color denotes the tissue in which the protein has been detected first. In NHP 24 spots are present in all the specimens examined. The comparison between NHP (Fig. 6A) and BPH (Fig. 6B) shows that 45 new proteins are present in BPH, while the cessation of the expression of only two species is observed. In PCa the increase in the Gleason score is accompanied by a dramatic increase in the complexity of the relative frequency diagrams. For Gleason score 4–5 21 new species have been detected, with relative frequencies between 0.12 and 0.37 (Fig. 6C). The subset of proteins originally detected in NHP undergoes limited quantitative changes; we observed the cessation of the synthesis of only two species. In the case of moderately differentiated tumors, one protein, which was already present in well-differentiated tumors, was found to be expressed in all the samples (NM-2 in Figs. 5D,E, 6B–E and Table IV) while 37 new species appear. Finally, in poorly differentiated tumors (Gleason score 8–9) five proteins (referred to as NM-3–NM-7 in Figs. 5E and 6B–E), which were already expressed in BPH (NM-3–NM-5)

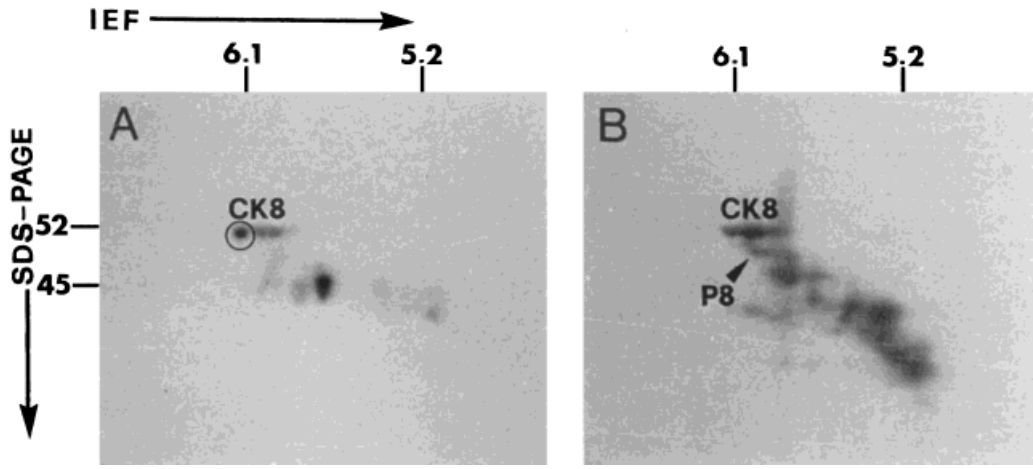


**Fig. 4.** Immunoblot characterization of the proteolytic degradation of CK8 in the NM-IF complex isolated from NHP (**A**) and PCa (**B**) using the monoclonal antibody 4.1.18. The proteolytic fragment P8 (arrowhead in B) was detected in all the PCas examined. The circle marks the more basic isoelectric variant of CK8.

and in PCa with Gleason score 4–5 (NM-3–NM-6) and 6–7 (NM-3–NM-7), were found in all the specimens examined (Fig. 6E), while there is the synthesis of 56 additional proteins. In a previous paper [Alberti et al., 1996] we have shown that a protein referred to as NM-1 was expressed in eight out of nine tumor samples having a Gleason score between 5 and 8. In this work we have found that this species is present in moderately and poorly differentiated tumors with a frequency of 0.78, while in well-differentiated PCas has been detected with a frequency of 0.5 (Fig. 5E).

These observations indicate that the development of less differentiated tumors is characterized by the stabilization of the expression of a few proteins which were already present in BPH or in tumors having a lower Gleason score. Therefore, our data confirm previous results by Partin et al. [1993] in support of a model of tumor progression in which BPH shares many of the changes in the NM proteins observed in PCa.

## DISCUSSION

In order to compare the results reported in this work with previous studies supporting the notion that the NM-IF is involved in the epigenetic modulation of tumor progression, it is appropriate to examine separately the nature of the changes the IF and NM proteins undergo in different tumors or in tumor cell lines.

### Alterations of the CK Pattern in Tumors

The correlation between the expression of simple epithelium type CKs and tumor progression is well documented. In an early study by Lifschitz-Mercer et al. [1991] the contents of simple CKs, especially CK19, in non-seminomatous germ cell tumors were found to be correlated with the level of morphological differentiation. Murant et al. [1997], using immunohistochemistry techniques, have shown that the expression of CK8 and CK18 is lost in poorly differentiated PCas; this loss is coordinate with changes in the expression of E-cadherin. Finally, a monotonical decrease in CKA and CKD, corresponding to human CKs 8 and 18, respectively, as well as the new expression of a simple keratin analogous to human CK19, have been observed in the resistant hepatocyte model [Barboro et al, 1996]. The results reported in this work are in line with the findings surveyed above: as a rule, the content of simple CKs decreases significantly in poorly differentiated carcinomas. Also in vitro experiments confirm this behavior: Prasad et al. [1998] have shown that SV-40 immortalized neonatal human prostate epithelial cells (267B1) undergo changes in the composition of the IFs during the neoplastic conversion induced by X-rays or by *ras*-oncogene; in particular these authors observed a stepwise down regulation of CK19. Down-regulation of simple CKs is not, however, invariably correlated with the malignant transformation: the evolution of

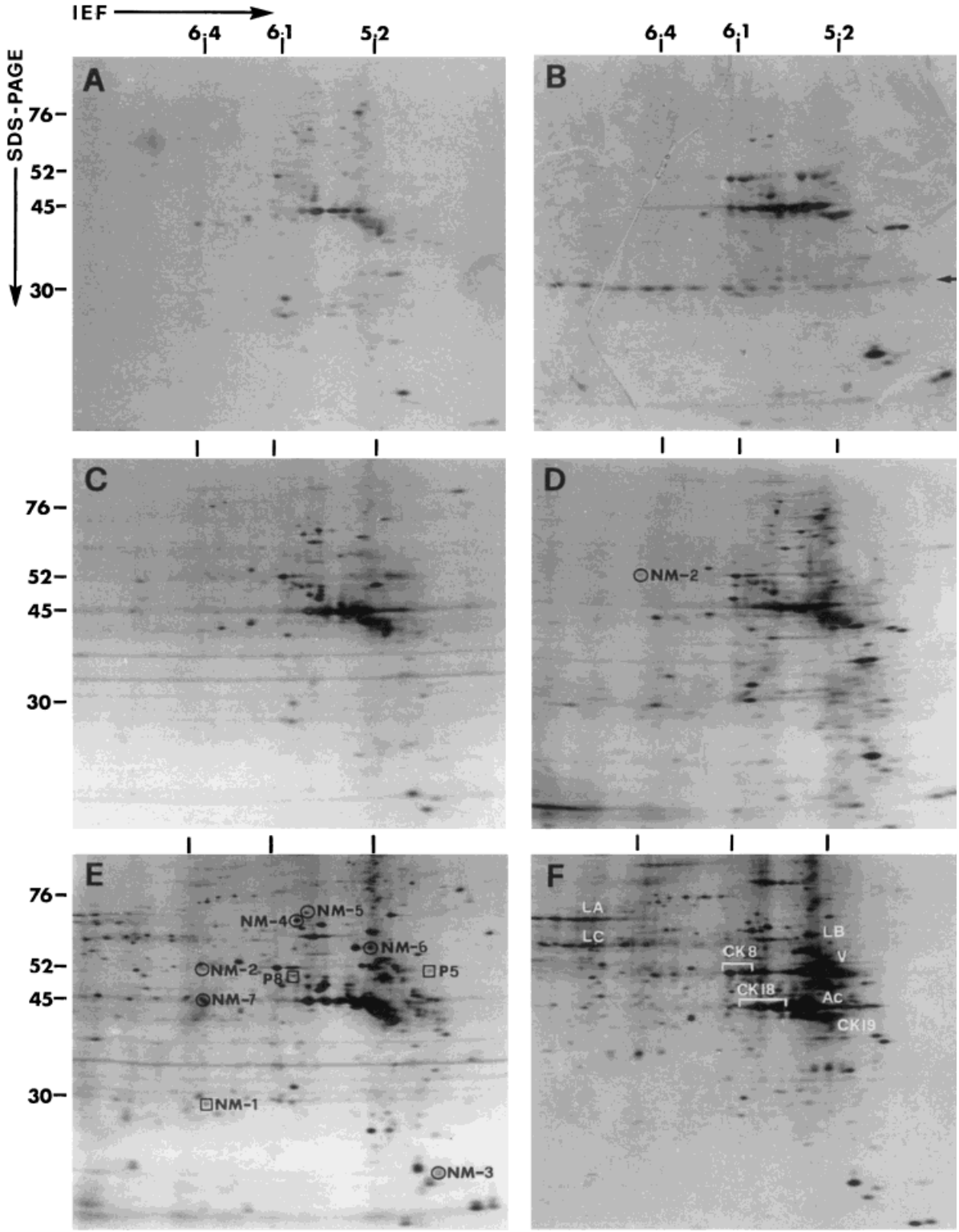


**Fig. 5.** High resolution two-dimensional gel electrophoresis of the NM-IF complex prepared from NHP (**A**), BPH (**B**), and PCas (**C–E**) with different Gleason score (4, 7, and 9, respectively). Silver stained gels. The new NM proteins expressed in all the tumor samples with a given Gleason score are marked by circles and identified with the same labels used in Table IV. The squares in E indicate the tumor-associated proteins (NM-1, P5 and P8) described in a previous report [Alberti et al., 1996]. Major NM-IF components are indicated in the gel reported in **F** (PCa with Gleason score 8). Lamins A, B, C actin and vimentin are designated by LA, LB, LC, Ac, and V, respectively. The position of the pI marker (carbamylated forms of carbonic anhydrase ) is indicated by an arrow in B.

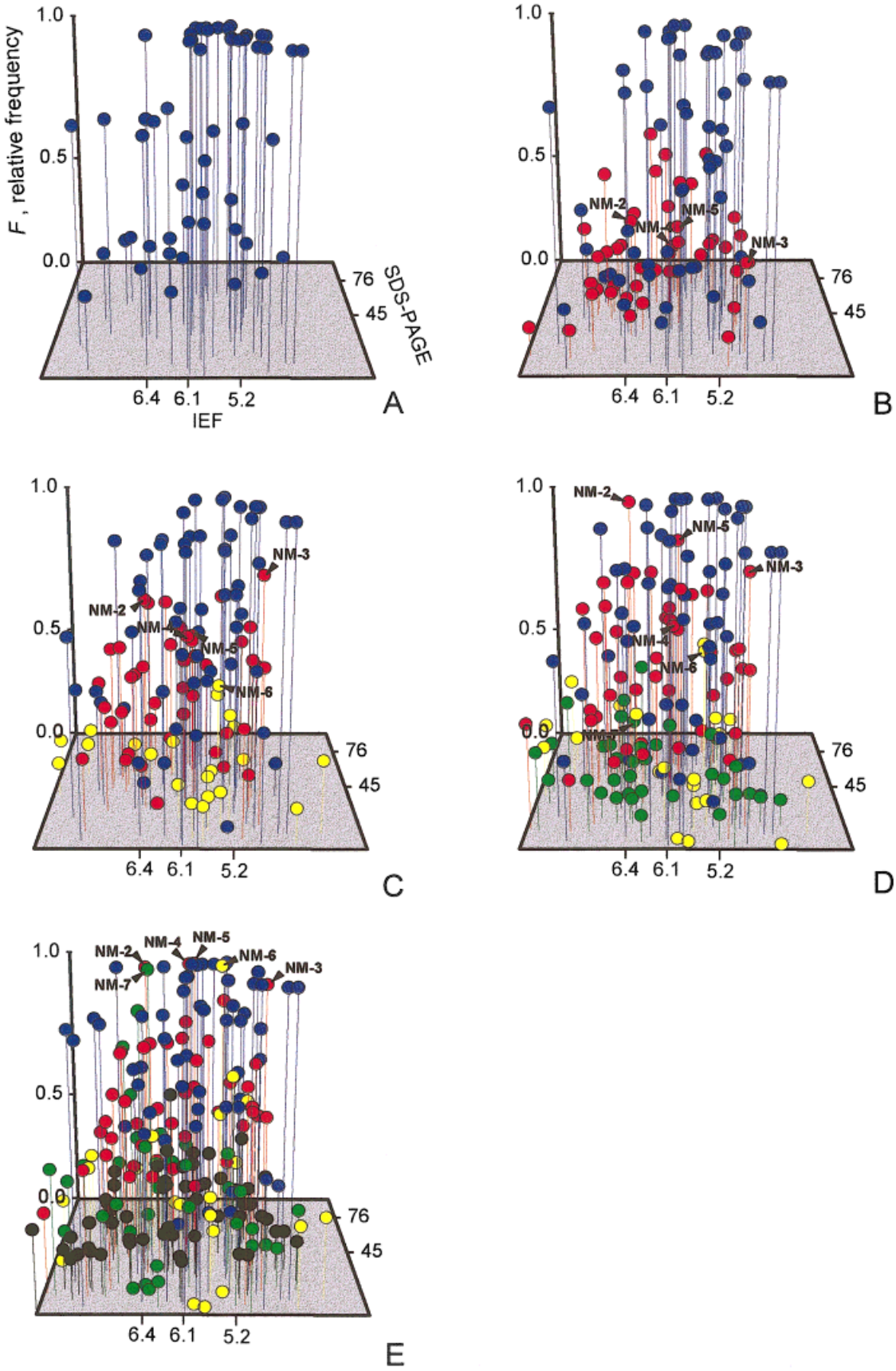


**Fig. 6.** Relative frequency diagrams of the NM proteins in NHP (**A**), BPH (**B**), and PCa with Gleason score 4–5 (**C**), 6–7 (**D**), and 8–9 (**E**). The lines indicate the positions of the protein spots in the IEF/SDS-PAGE plane and heights represent the relative frequencies with which the proteins occur in the different tissues. The color denotes the tissue in which the proteins has been detected first (blue, NHP; red, BPH; yellow, green, and black, PCas with Gleason score 4–5, 6–7, and 8–9, respectively). NM proteins correlating with the Gleason score are marked as in Table IV.

**TABLE IV. Molecular Characterization and Relative Frequency of the NM Proteins Related to Tumor Differentiation in NHP, BPH and PCas With Different Gleason Score**

| | | | | | PCa | | |
|---|---|---|---|---|---|---|---|
| Protein | $M_r \times 10^{-3}$ | p.I. | NHP | BPH | Gs[a] 4–5 | Gs 6–7 | Gs 8–9 |
| NM-2 | 51 | 6.40 | 0 (0/5)[b] | 0.31 (5/16) | 0.66 (4/6) | 1 (13/13) | 1 (7/7) |
| NM-3 | 20 | 4.90 | 0 (0/5) | 0.31 (5/16) | 0.83 (5/6) | 0.84 (11/13) | 1 (7/7) |
| NM-4 | 66–68 | 5.90 | 0 (0/5) | 0.18 (3/16) | 0.50 (3/6) | 0.54 (7/13) | 1 (7/7) |
| NM-5 | 67–69 | 5.80 | 0 (0/5) | 0.25 (4/16) | 0.50 (3/6) | 0.85 (11/13) | 1 (7/7) |
| NM-6 | 57 | 5.35 | 0 (0/5) | 0 (0/16) | 0.33 (2/6) | 0.46 (6/13) | 1 (7/7) |
| NM-7 | 46 | 6.35 | 0 (0/5) | 0 (0/16) | 0 (0/6) | 0.23 (3/13) | 1 (7/7) |

[a]Gs, Gleason score.
[b]Number of positive samples/number of cases examined.

breast epithelial cells towards cancer involves an increase in the expression of CKs 8 and 18 [Trask et al., 1990]. Furthermore, the ectopic induction of the latter in tumors has been in some cases observed [Oshima et al., 1996].

Another remarkable demonstration of the involvement of the status of the CKs in the evolution towards the malignant phenotype is the occurrence of an extensive fragmentation process induced by $Ca^{2+}$ dependent, CK specific proteases. In all the PCas examined, CK8 exhibits a sharp staircase pattern of fragments in two-dimensional gels. In a study on the adenocarcinoma of the colon, two out of six tumor-associated proteins have been shown to be homologous with CKs 18 and 19 by partial aminoacid microsequencing, and could, therefore, represent fragments of these species [Keesee et al., 1996]; moreover, epitopes of CKs 8, 18, and 19 were found at elevate levels both in the serum of patients with prostate cancer [Tarle, 1993] and in a wide range of carcinomas [van Dalen, 1996].

Taken together, these findings support the view that structural rearrangements of the CK filaments induced by changes in the protein composition or possibly, the activation of CK specific proteases play a direct role in carcinogenesis. Recently, Davie ad his colleagues [Spencer et al., 1998] were able to extend previous observations by Olinski et al. [1987] on the association of CKs with nuclear DNA. In a hormone-dependent and estrogen receptor-positive human breast cancer cell line, the levels of CKs 8, 18, and 19 are regulated by estrogens [Coutts et al., 1996]. When the cells were treated with *cis*-diamminedichloroplatinum to crosslink proteins to nuclear DNA in situ, CKs 8, 18, and 19 were found to be associated with DNA. The levels of the CKs bound to nuclear DNA or associated with the cytoskeleton decrease as a consequence of estrogen withdrawal or anti-estrogen administration. This result suggests that rearrangement of the CK intermediate filaments triggers the reorganization of chromatin [Spencer et al., 1998]. The same mechanism has been put forward to explain the occurrence of a temporal correlation between CK changes and chromatin decondensation during the transformation of rat hepatocytes [Barboro et al., 1996]. Basically the structural rearrangement of the cytoskeleton, resulting from an altered content of IF proteins, might offer a distinct growth advantage to the transformed cell. We have recently found that in rat liver nodules the cellular subpopulation which ectopically expresses CK19 has a rate of proliferation much higher than that of the other (CK19 negative) transformed hepatocytes (unpublished results).

While the differences in the expression of CKs 8, 18, and CK19 among the PCas of different grade, although significant, have no practical diagnostic utility, the presence in all of the PCas examined of a fragment of CK8 referred here to as P8 (Fig. 4) could represent a valuable biomarker and therefore deserves further molecular characterization.

### NM Protein Patterns in PCa and Tumor Heterogeneity

Genetic instability is believed to determine tumor heterogeneity, which in turn represents a major engine of tumor progression [Lengauer et al., 1998]. In a recent commentary on self-organization, complexity, and chaos, Coffey [1998] has advanced the concept of the chaotic nature of cancer development; the onset of ge-

netic instability produces a tremendous diversity, which ultimately may lead to cancer. Instability is observed both at the nucleotide and at the chromosome level. Abnormal translocations and genomic rearrangements frequently involve DNA sequences at the base of chromatin loops [Boulikas, 1992] and can therefore be mediated by the interaction between DNA and the NM.

The dramatic broadening which occurs in the relative frequency diagrams with increasing the Gleason score (Fig. 6) suggests that heterogeneity plays a central role also in the progression of PCa. It is interesting to note that, as the broadening reflects the increase in the phenotypic diversity arising from the emergence of different cellular subpopulations, the stages of tumor development hardly could be characterized using single marker proteins. For example, the frequency data reported in Table IV show that the expression of NM-7 can be used to distinguish between low (4–5; the protein was absent in all of the samples examined) and high (8–9) Gleason (the protein was found in all the samples examined); the fact that this species occurs in three out 13 samples with intermediate Gleason, however, does not allow to distinguish between low and intermediate as well as between high and intermediate Gleason. Instead, the correlation between the frequency data and Gleason score can be obtained by considering a set of tumor-associated proteins rather than a single species. If $n$ proteins are expressed independent of each other, the probability $P$ of these proteins being coexpressed is simply the product of the individual probabilities. In tumor samples of intermediate Gleason (Table IV) proteins NM-3, 4, 6, and 7 are expressed with relative frequencies of 0.84, 0.54, 0.46, and 0.23, respectively. If we assume that the probabilities are not much different from the experimental frequencies, we obtain $P = 0.05$. This value is much less than 1, so making possible to distinguish between intermediate and high Gleason ($P = 1$).

While this simple result suggests that the statistical analysis of the heterogeneity of the NM proteins should be further pursued, in that it is expected to yield useful parameters for the molecular characterization of the tumor stage, the question arises whether or not the two-dimensional electrophoretic characterization of NM proteins represents an adequate starting point for the identification of species involved in the molecular mechanisms of carcinogenesis. As far as this methodological point is concerned, Nickerson [1998] has recently pointed out that even if we knew the identity of malignancy specific protein spots, it would be difficult to study their role in inducing tumor specific changes in the nuclear architecture. This statement has an important element of truth, because it is quite plausible that also rearrangements in the nuclear scaffold occur in a coordinated fashion with the cytoskeletal changes, and represent therefore an early event in transformation. Nevertheless, we cannot absolutely rule out that changes in other NM proteins are needed in order to induce unfolding of the higher order structure of chromatin; a few tumor associated proteins might be components of the machinery of chromatin opening. We have recently shown that the anchoring of the polynucleosomal chain to two sites of the nuclear scaffold represents a necessary condition for the stabilization of heterochromatin [Balbi et al., 1999]. Therefore, the number of copies per nucleus of a hypothetical matrix attachment region (MAR) binding protein involved in anchoring is expected to be twice the number of loops. The latter is unknown for prostate epithelial cells but we can rely on the values obtained for HeLa cells ($3.3 \times 10^5$ and $6.4 \times 10^4$, respectively, depending on two different estimates of the loop size) [van Holde, 1989]. If we assume that the protein has a molecular weight of 100,000 its weight amount in the nucleus will be between 0.02 and 0.1 pg. Since in a typical two-dimensional electrophoresis the NM-IF complexes isolated from about $3 \times 10^6$ cells are run on the gel, the amount of the protein submitted to electrophoresis will be in turn between 0.06 and 0.3 μg, a figure which is actually well above the detection limit of the silver staining. If these considerations are correct, we can speculate that relevant information on the molecular mechanisms of cancer development is still hidden in our standard two-dimensional electrophoretic gels of NM preparations and could be gained by enhancing the specificity of the Southern Western blot analysis using appropriate MAR sequences.

## ACKNOWLEDGMENTS

We thank Dr. David Malacarne for statistical analysis and R. Fiorini for excellent technical assistance. This work was supported by

A.I.R.C. (Associazione Italiana per la Ricerca sul Cancro), Ministero della Sanità, and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST)

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