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Ras-Associated Nuclear Structural Change Appears Functionally Significant and Independent of the Mitotic Signaling Pathway

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Abstract An altered nuclear morphology has been previously noted in association with Ras activation, but little is known about the structural basis, functional significance, signaling pathway, or reproducibility of any such change. We first tested the reproducibility of Ras-associated nuclear change in a series of rodent fibroblast cell lines. After independently developing criteria for recognizing Ras-associated nuclear change in a Papanicolaou stained test cell line with an inducible H(T24)-Ras oncogene, two cytopathologists blindly and independently assessed 17 other cell lines. If the cell lines showed Ras-associated nuclear change, a rank order of increasing nuclear change was independently scored. Ras-associated nuclear changes were identified in v-Fes, v-Src, v-Mos, v-Raf, and five of five H(T24)-Ras transfectants consisting of a change from a flattened, occasionally undulating nuclear shape to a more rigid spherical shape and a change from a finely textured to a coarse heterochromatic appearance. Absent or minimal changes were scored in six control cell lines. The two cytopathologists' independent morphologic rank orders were similar ($P < .0002$). The mitogen signaling pathway per se does not appear to transduce the change since no morphologic alterations were identified in cell lines with activations of downstream components of this pathway—MAPKK or c-Myc—and the rank orders did not correlate with markers of mitotic rate ($P > .11$). The rank order correlated closely with metastatic potential ($P < .0014$ and $P < .0003$) but not with histone H1 composition or global nuclease sensitivity. Based on published studies of five of the cell lines, there may be a correlation between increases in certain nuclear matrix proteins and the Ras-associated nuclear change. J. Cell. Biochem. 70:130–140, 1998.

Key words: signal transduction; chromatin structure; cytology; histones; metastasis; Ras; MAPKK; NIH3T3 cells

A basic theorem underlying diagnostic cytopathology is that the functional state of a cell is predictably reflected in the structure of the nucleus [Frost, 1986]. Thus, activated lymphocytes can be distinguished from resting lymphocytes, and benign cervical cells can be distinguished from malignant cervical cells by nuclear morphologic features. Since oncogenes alter the functional state of cells, it seems reasonable for predictable large-scale nuclear morphologic/structural changes to be associated with oncogene activation. To begin to test this hypothesis, we previously examined a Rat-1 fibroblast cell line containing a transfected zinc-inducible H(T24)-Ras oncogene (MR4 cells) and quantified major changes in nuclear morphology by image analysis [Fischer et al., 1992]. Similar ideas have been explored by others [Mello and Russo, 1990; Mello and Chambers, 1994]. The present study was designed to test the reproducibility of this phenotype, to learn structural and functional correlates of any observed nuclear changes, and to try to make deductions about the signaling pathway for its transduction.

Understanding signaling pathways from receptor tyrosine kinases (RTK) has been of great interest in the hope that novel cellular targets involved in carcinogenesis may be disclosed. A problem with the signal transduction paradigm is that it is not obvious what pathways should

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be followed. The pathway leading to increased DNA synthesis and mitogenesis has been most intensely investigated [reviewed by Avruch et al., 1994; Egan and Weinberg, 1993], although there are numerous other pathways that could be followed depending on the cell type and specific RTK, including increased glucose and amino acid transport, ion exchanges, calcium influx, cytoskeletal changes, altered in vitro growth characteristics, and various changes in differentiation [reviewed by Cantley et al., 1991; Ullrich and Schlessinger, 1990]. It is possible to trace a mitogenic signal from RTKs (occasionally through nonreceptor tyrosine kinases including Src and Fes) to Ras to the serine/threonine kinases Raf or Mos to a mitogen-activated protein kinase kinase (MAPKK, also known as MEK) to mitogen activated protein kinase (MAPK) and finally to nuclear targets consisting of various transcription factors and nuclear oncogenes (including c-Myc) [Avruch et al., 1994; Smith et al., 1986]. However, the importance of this mitogen signal transduction pathway in carcinogenesis is uncertain: cancer cells often have a longer cell cycle than their normal counterparts [Cotran et al., 1994], for example, and naturally occurring mutant MAPKK or MAPK mutations have not been identified in human cancers [Cowley et al., 1994].

Instead of following a mitogenic signal, a variety of changes in the actin cytoskeleton can be traced back to RTK activation [reviewed by Chant and Stowers, 1995]. Since some cytoskeletal changes correlate with tumorigenicity [reviewed by Hanafusa, 1977; Pastan and Willingham, 1978], there has been interest in understanding their precise structural basis. However, the few specific alterations (membrane ruffles and stress fiber formation/focal adhesions) successfully traced through Ras superfamily members Rac and Rho [Ridley et al., 1992; Ridley and Hall, 1992] are of uncertain significance to malignancy [discussed by Prendergast et al., 1995], since activation of these pathways are at most only minimally transforming [Perona et al., 1993].

In contrast to cytoplasmic changes, little is known about nuclear morphologic changes associated with activation of RTKs or their downstream effectors [Fischer et al., 1992; Mello and Russo, 1990; Mello and Chambers, 1994], even though malignant cells are diagnosed on the basis of nuclear morphologic abnormalities [Barker and Sanford, 1970; Frost, 1986; Sanford et al., 1970]. Histopathology studies have typically sought correlations between oncogene expression and low magnification architectural features or degree of differentiation rather than with specific cytologic features [Nishio et al., 1992].

MATERIALS AND METHODS

Cell Lines

MR4 cells (a gift of Michael Lieberman) [Reynolds et al., 1987b] are Rat-1 cells stably transfected with a metallothionein-H(T24)-Ras construct. $ZnSO_4$ (100 μM) induces four- to fivefold stimulation of Ras transcripts within three days [Reynolds et al., 1987b]. 2H1 cells are 10T1/2 cell lines with a stably transfected metallothionein-H(T24)-Ras [Haliotis et al., 1990]. Ras transcripts increase twofold by 12 h of induction with 50 μM $ZnSO_4$ [Haliotis et al., 1990].

Ciras-1, Ciras-2, Ciras-3, and NR4 cells are stable H(T24)-Ras transfectants of 10 T1/2 cells [Egan et al., 1987a]. NIH3T3 cells transformed with human c-Myc, v-Mos, v-Fes, v-Src, or A-Raf are named NIH/hmyc1, Mos 1, Fes 1, Src 1, and NIH/9IV#5, respectively [Egan et al., 1987b]. The metastatic potential of the Ciras 1-4, NR4, NIH/hmyc1, Mos 1, Fes 1, and NIH/9IV#5 cell lines have been previously determined [Egan et al., 1987a,b] by injecting 3×10^5 cells into the lateral tail vein of 5–8-week-old BALB/c female nu/nu mice and counting the number of lung nodules after 3 weeks.

NIH3T3 cells with transfected inactive MAPKK or transfected and transformed with constitutively active MAPKK are K97 and ΔN3-S222D, respectively [Mansour et al., 1994] (a gift from Drs. Natalie Ahn and Sam Mansour).

Control cell lines are the parental Rat-1 fibroblast cells of the MR4 line and MR4 cells grown without Zinc induction, 10T1/2 and NIH3T3 parental cell lines of the above transfectants [Egan et al., 1987a,b], NIH3T3 cells transfected with wild-type MAPKK and vector only [Mansour et al., 1994], and 2H1 cells grown without Zn induction. The parental 10T1/2 and NIH3T3 lines are mouse fibroblast in origin and are aneuploid but nontumorigenic. The Rat-1 cells are weakly tumorigenic and also aneuploid [Reynolds et al., 1987a]. Histone H1 subtype levels, histone H1 phosphorylation status, and measurement of general nuclease sensitivity have been published for these same passage numbers [Chadee et al., 1995].

Culture Conditions

MR4 and Rat-1 cells (2×10^4) were plated in 1 ml DMEM supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) on glass chamber slides (lab-tech chamber slides, two chambers; Nunc, Naperville, IL) supplemented with penicillin and streptomycin with 7% CO_2 at 37°C. One day later, $ZnSO_4$ was added to 100 μM final concentration to some groups of MR4 or control Rat-1 cells. Cells were then grown for 1 or 3 more days before fixation. To assess the reversibility of the change, we passaged and grew some MR4 cells for 3 days in Zn-free media after growing it for 3 days with 100 μM Zn.

All other control and oncogene-transfected cells were plated and grown under identical conditions on glass chamber slides at 37°C with 7% CO_2 in alpha-MEM plus 10% fetal bovine serum (Intergen, Purchase, NY). 2H1 cells were induced with 50 μM $ZnSO_4$ for 12 h.

Cytopathology Assessment

All cells were fixed before confluence by immersion in 95% ethanol without any air-drying before or after fixation. A modified Papanicolaou stain (Harleco, Gibbstown, NJ) (the same stain used for diagnosing human clinical samples) was used. MR4 cells and Rat-1 cells were examined independently by two experienced cytopathologists (A.H.F. and T.S.G.) aware of the genetic makeup of the cell line in order to develop criteria for recognizing the Ras-associated nuclear change. All the other cell lines including controls were sent coded for Papanicolaou staining and blinded independent study by the two cytopathologists 1) to determine if the cells showed Ras-associated nuclear change similar to the Ras-expressing MR4 cells, and, if so, 2) to rank the order of increasing Ras-associated nuclear change in the cell lines by performing multiple paired comparisons between cell lines.

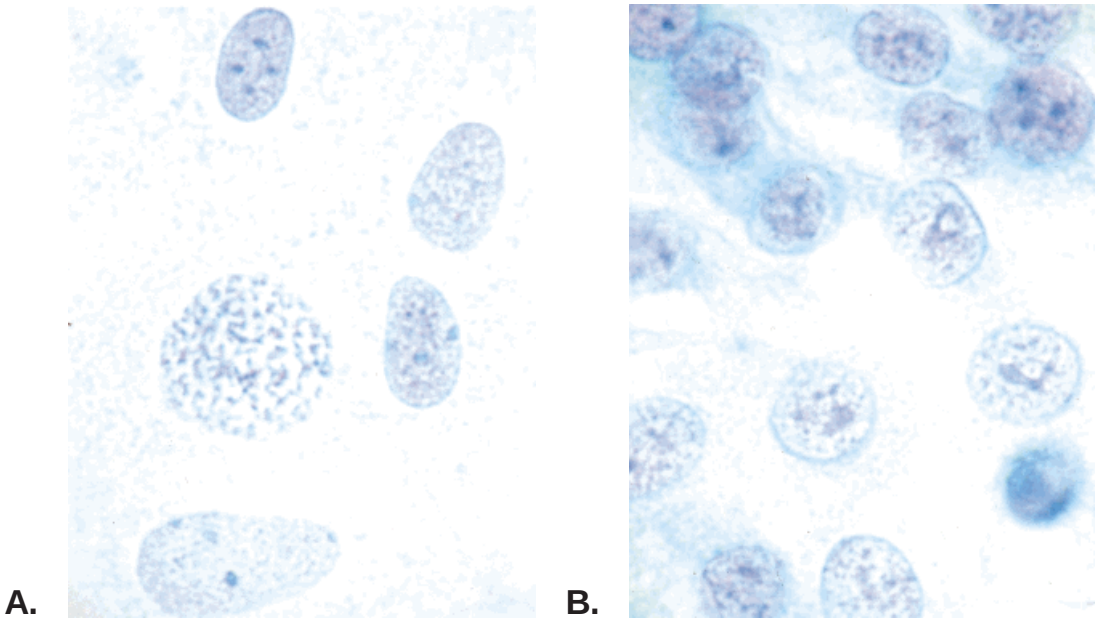
RESULTS

Appearance of Parental Rat-1 Cells Compared to MR4 Cells: Description of a Ras-Associated Nuclear Change

The independently derived descriptions of the nuclear features of the Rat-1 parental cell lines (Fig. 1A) by the two cytopathologists include a flattened nucleus toward the substrate, an oval to round nuclear contour with occasional smooth undulations, moderate variability in nuclear and nucleolar size, and fine textured chromatin with no large areas of euchromatin. The zinc sulfate used to induce Ras expression has no effect on cytomorphology (not shown). MR4 cells grown without zinc sulfate show a slightly altered nuclear appearance compared to Rat-1 cells that may be related to a slight leakiness of the metallothionein promoter [Reynolds et al., 1987b]. MR4 cells grown in the presence of 100 μM zinc for just 1 day are not altered in morphology. However, after 3 days of Zn induction, MR4 cells show a dramatic change in nuclear morphology (Fig. 1C; same magnification as Fig. 1A) characterized by cytopathologist 1 as more rigidly spherical nuclear shape, occasionally with nuclear membrane clefts or grooves, and having increased coarseness of the chromatin while it remains symmetrically distributed and without large areas of euchromatin. Cytopathologist 2 independently characterized this Ras-associated nuclear change as a change to a more uniformly sized spherical nucleus with increased chromatin coarseness and hyperchromasia. The morphology of the induced MR4 cells reverts after replating the cells without zinc and growing them for 3 days (Fig. 1B). The difference in appearance between Ras expressing and nonexpressing MR4 cells can be objectively distinguished by the Cas (Elmhurst, IL) 100 system cell measurement program on feulgen-stained nuclei [Fischer et al., 1992, unpublished data].

Varying Degrees of Development of Ras-Associated Nuclear Change Are Seen in Many Oncogene Transfectants

Cell lines bearing V-Fes, v-Src, a-Raf, v-Mos, and four of five cell lines expressing H(T24)-Ras were judged blindly and independently by both cytopathologists to show nuclear features similar to MR4 cells (Fig. 2B–I; Table I). By contrast, neither cytopathologist identified any nuclear morphologic change in the c-Myc transfected line (Fig. 2A) or in the line containing constitutively active MAPKK (Fig. 3A). The two cytopathologists each judged four of six coded control cell lines to show nuclear features similar to the Rat-1 parental control line; one cytopathologist declined judgment about 10T1/2 control cells due to the presence of a rare group of cells with three-dimensional growth and complex nuclear membrane contours and also judged the parental NIH3T3 cells to show minimal coarsening (Table I); the other cytopatholo-



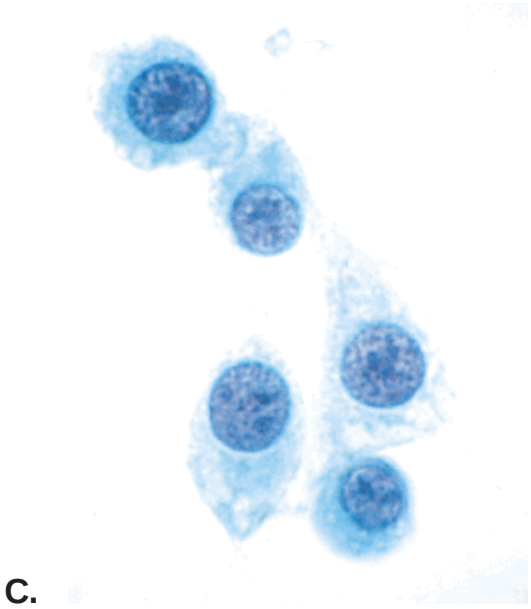


Fig. 1. Appearances of the cell lines used for developing criteria for Ras-associated nuclear change. Parental rat-1 fibroblast cells (**A**) and MR4 cells (**C**) are shown after growing in the presence of zinc for 3 days. Panel **B** shows MR4 cells reverted by replating for 2 days in zinc-free media. All cells are alcohol-fixed without air drying and shown with Papanicolaou staining at equivalent magnification. Field of view is 25 × 33 microns. Note late prophase mitosis in Rat-1 cell (A).

gist felt that two controls (one NIH3T3 cell line transfected with wild-type MAPKK and one control NIH3T3 line transfected with neomycin resistance) showed minimal Ras-associated nuclear change (Table I). After the genetics of the cell lines were known, review showed slightly smaller nucleoli in the c-Myc line compared to the other control lines as the only nuclear change in this line, but no morphologic clue to the presence of a constitutively active MAPKK could be seen on review. Figure 3B shows the appearance of the K97 cell line transfected with a constitutively inactive MAPKK.

The lines differed from each other in the degree of Ras-associated nuclear change, and multiple side-by-side comparisons allowed a rank order of increasing average Ras-associated nuclear change to be determined independently by both cytopathologists (Table I). Through such pairwise comparisons, one cytopathologist separated eight groups of increasing change, the other seven. The rank order for the two independent observers is similar (Fig. 4A) (correlation coefficient = 0.82; probability that the two rank orders are random with respect to each other is <0.0002).

Rank Order of Increasing Ras-Associated Nuclear Change Correlates With Metastatic Potential But Does Not Correlate With S-Phase Fraction, Overall Sensitivity of the Nuclei to Nuclease Digestion, or Histone H1 Composition of the Cell Lines

The rank order of increasing Ras-associated nuclear change in the cell lines correlates closely with their previously measured metastatic potential (Fig. 4B) (probability that the rank orders of increasing nuclear change is random with respect to metastatic potential is 0.0014 and 0.0003 for the two cytopathologists). Previous measure of Ras expression in some of these cell lines has shown a positive correlation with metastatic potential [Egan et al., 1987a], and thus it would appear that the degree of expression of Ras should correlate with the degree of Ras-associated nuclear change.

The degree of Ras-associated nuclear change is independent of the mitotic rate as determined by mitotic counts, image analysis of S-phase fraction (in MR4 cells) (data not shown), and flow cytometry (Table I). There is no relation between the nuclear appearance and the degree of confluence of the cells. The appearance of a prophase mitosis of a parental cell line can be distinguished from the appearance of the transformed lines (e.g., Fig. 1A). The rounding of the nucleus and coarsening of chromatin in the MR4 and other transformed lines shows some resemblance to the appearance of postmitotic cells as the chromosomes decondense, but postmitotic cells of the transformed lines retain a morphology distinctly different from their respective interphase cells.

The histone composition of most of these same cell lines has recently been reported [Chadee et al., 1995]. Since histone H1 mediates chromatin packaging at a supranucleosomal level [Cole, 1987; Davie, 1996], changes in histone H1 composition are implicated in large-scale chromatin organization. However, neither the level of total histone H1 compared to the core histones nor the proportions of five histone H1 subtypes separable by two dimensional electrophoresis (H1o, H1a, H1b, H1c, and H1d) predict the presence of oncogene activation or correlate in any obvious manner with the nuclear morphologic changes of these cell lines [Chadee et al., 1995]. Histone H1 undergoes a variety of covalent modifications [Cole, 1987; Davie, 1996], and one modification found to correlate with the presence of transfected oncogenes is phosphorylation [Chadee et al., 1995; Taylor et al., 1995]. The level of one form of phosphorylated H1b that can be measured relatively easily, and which tends to reflect the levels of the other phosphorylated H1 subtypes, is shown in the last column of Table I [Chadee et al., 1995]. The global level of this phosphorylated H1b subtype bears no clear relation to the nuclear features of the cell lines. For example, both the NIH/hmyc1 (c-Myc) and ΔN3-S222D-NIH3T3 (MAPKK) show no change in morphology despite increased phosphorylated H1 levels comparable to the Ras or Src transformed lines Ciras-2 and Src-1 (compare Figs. 2A and 3A with Fig. 2F,H).

Ciras-2 and Ciras-3 cells showed greater general sensitivity to exogenous nuclease digestion compared to control 10T1/2 cells, as manifest by a faster release of smaller DNA fragments in a standardized assay [Chadee et al., 1995]. Similar results for other Ras-transfected cell lines have been reported [Laitinen et al., 1995b]. However, the NIH/hmyc1 line—devoid of Ras-associated nuclear change—showed a similar degree of increased sensitivity to nucleases as the Ras transfectants [Chadee et al., 1995], suggesting that there is no clear relation between measures of general nuclease sensitivity and the observed large-scale nuclear morphologic changes.

Other Miscellaneous Observations

There was minimal cell-to-cell variation in nuclear morphology within the cell lines of MR4 cells, Mos-1, NIH/9IV#5 (a-Raf), and Ciras-2 (Figs. 1C, 2D,E,G, respectively). Mild cell-to-cell variation was seen in the Fes-1 line. The Ciras-3, Src-1, Ciras-1, and NR4 showed moderate cell-to-cell variation in the degree of rounding of the nucleus and coarsening of the chromatin.

The Ciras-3 line (with an activated H-Ras) appeared different from the other cell lines to one of the two cytopathologists (Fig. 2H), who

Fig. 2. A–I: Correlation of nuclear morphology with metastatic potential. Shown in order of increasing metastatic potential from A to I are NIH/hmyc1 (expressing c-myc), NR4 cells, Ciras-1, Mos 1, NIH/9IV#5 (Raf-expressing), Src-1, Ciras-2, Ciras-3, and Fes-1. Table I gives their metastatic ability and morphologic rank order. All cells were fixed, Papanicolaou-stained, photographed, and printed under identical conditions at ×2,000 original magnification. Two rhabdoid cells are indicated by arrows in the v-src–transfected cell line (F) (see text).

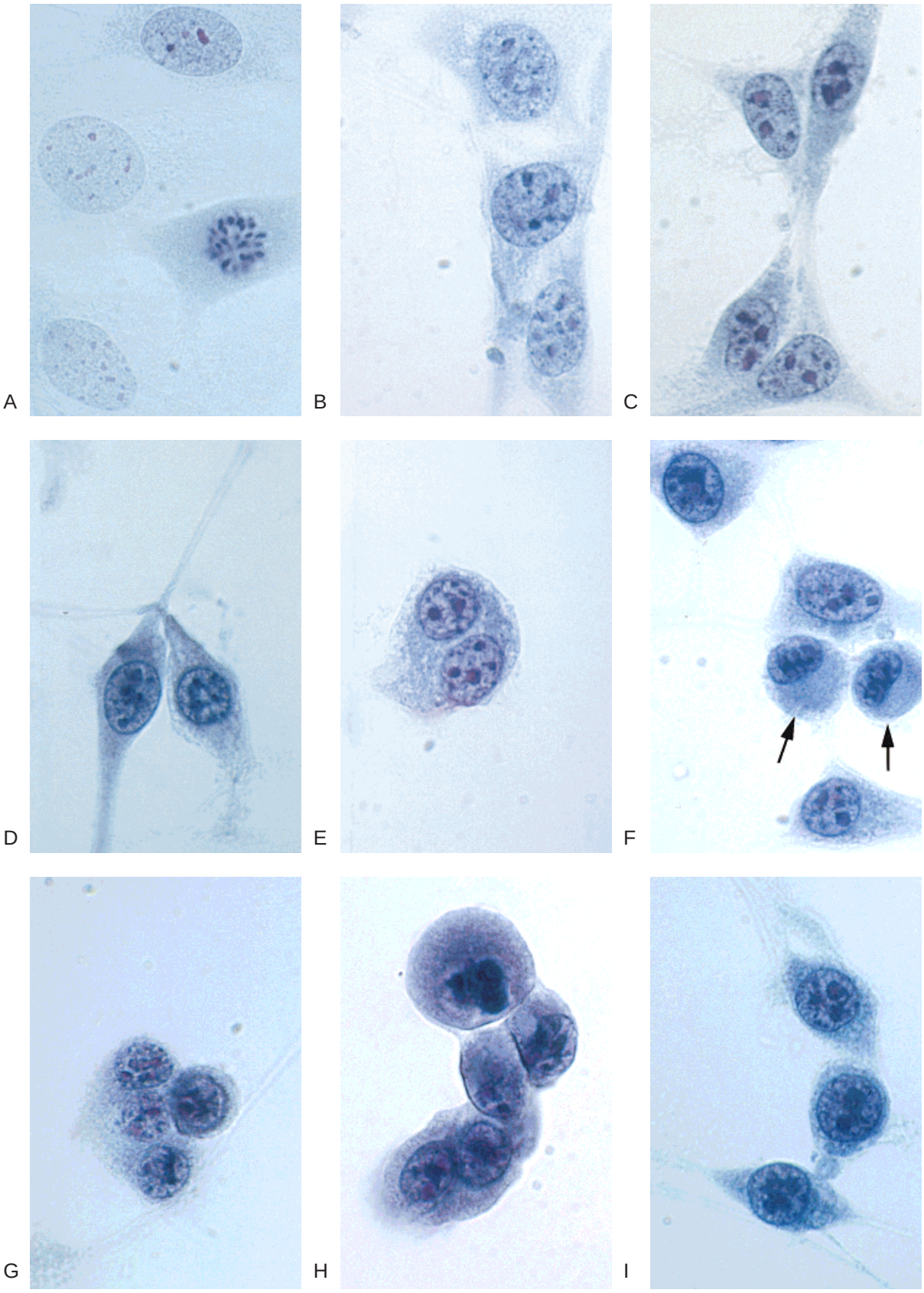


Figure 2.

noted much more cell-to-cell variation in the degree of chromasia and chromatin patterns, frequent deep and unpredictable nuclear membrane folds, and occasional cells with large areas of open chromatin. This highly aggressive-appearing line also shows large numbers of rhabdoid cells (i.e., cells with a dense rounded aggregate of homogeneously staining material, presumably intermediate filaments, adjacent to the nucleus. Cells with a rhabdoid appearance are also noted in large numbers in the v-Src line (Fig. 2F, arrows). The v-Mos line (Fig. 2D) tends to have long, bipolar cell processes.

DISCUSSION

Katherine Sanford [Sanford et al., 1970] and Barbara Barker [Barker and Sanford, 1970] found in 1970 that they could accurately assess tumorigenicity of cells in culture with stains and cytologic criteria used for diagnosing human cancers. To the best of our knowledge, their accuracy in predicting tumorigenicity (93% in a large study and 100% in a smaller group) remains superior to any biochemical test or in vitro assay of tumorigenicity. Their results are obscure, probably because nuclear structure appeared unapproachable in the 1970s.

We have been interested in testing the hypothesis that oncogene function is in some cases associated with predictable changes in nuclear structure. The hypothesis is suggested by the fact that cancer cell nuclei are usually grossly altered compared to normal cells (the alteration in appearance permits their diagnosis), yet the altered morphology cannot be adequately explained by aneuploidy or an increased growth rate [Frost, 1986]. Distinctive nuclear morphologic changes diagnostic of some tumors are often conserved during tumor evolution. Support for the hypothesis that the altered nuclear morphology of cancer cells is sometimes related to oncogene function comes from the association of certain oncogene activations with certain morphologically distinctive tumor types (e.g., the unique association of the papillary thyroid carcinoma oncogene with papillary thyroid carcinomas [Santoro et al., 1992]). The idea is also consistent with the notion that large-scale organization of heterochromatin and euchromatin may reflect the transcriptional program of a cell [Lawrence et al., 1993], and the transcriptional program in turn may be set by oncogenes. Although a plethora of cytologic changes was anticipated by the cytopathologists, perhaps reflecting the variety of cytologic appearances of various tumors, most of the cell lines had varying degrees of the Ras-associated change first observed in the MR4 cells: transformation to a more rigidly spherical nuclear shape and increasing coarseness/chromasia of a fairly uniformly distributed chromatin. The appearance is familiar, similar to the coarse chromatin cytopathologists describe in a variety of human tumors [Frost, 1986]. Two cell lines expressing distal components of the mitogen signal transduction pathway did not show any change in chromatin morphology, and one Ras-transfected cell line was felt by one cytopathologist to be unlike the other lines in that chromatin distributions were asymmetric, abundant euchromatin was present, and nuclear membrane contour irregularities were seen. It will be interesting to see if the nuclear membrane folds and deep clefts observed in this one line are related to the function of another oncogene. Recently Waldman et al. [1996] noted nuclear polylobulation in cells lines defective in p53 and p21.

Virtually nothing is known about the structural basis for the nuclear morphologic changes. Our study allows inferences about the roles of histone modifications, changes in nuclease sensitivity, and distal components of the mitogen signal transduction pathway. Histone H1 subtypes may be expected to be related to the change since they vary in a tissue- and differentiation-specific manner, they mediate supranucleosomal-level chromatin packaging, and they have a nonrandom interphase distribution [Cole, 1987; Davie, 1996]. However, no clear correlation between global histone H1 phosphorylation or subtype levels and nuclear morphology is evident. Of course, our analysis does not exclude the possibility of an altered distribution of histone H1 subtypes. A lack of correspondence between global H1 histone phosphorylation and metastatic potential has also been observed in other cell lines [Taylor et al., 1995].

NIH3T3 cells transfected with Ras display an increased sensitivity to nuclease digestion [Chadee et al., 1995; Laitinen et al., 1995b]. However, the change in heterochromatin texture in the various cell lines bears no simple relation to the overall sensitivity of the chromatin to exogenous nucleases in these cells. DNA methylation may not be involved in the large-scale chromatin alteration associated with Ras transformation; Laitinen et al. [1995a] found no changes in global DNA methylation or meth-

TABLE I. Rank Order of Metastatic Potential Correlates With the Rank Order of Ras-Associated Nuclear Change (See Text and Fig. 4) for Two Independent Cytopathologists But Does Not Correlate With Global Levels of Histone H1 Phosphorylation or S-Phase Fraction

Cell line (oncogene)	Metastatic potential[a] or tumorigenicity[b]	Rank order of increasing Ras- associated change		Phosphorylated histone H1b level compared to control cells[c]	% S-phase fraction[c]
		Cytopathologist 1	Cytopathologist 2		
Fes-1 (NIH3T3 cells with v-Fes)	178 ± 38[a]	7	7	3.5	26
Ciras 3 (10T1/2 cells with H(T24)-Ras)	121 ± 20[a]	Not applicable[d]	7	4.2	22
Ciras 2 (10T1/2 cells with H(T24)-Ras)	118 ± 6[a]	8	6	3.8	34
Src-1 (NIH3T3 cells with v-Src)	59 ± 25[a]	6	6	Not done	Not done
NIH/9IV#5 (NIH3T3 cells with a-Raf)	38 ± 14[a]	5	2	1.7	27
Mos-1 (NIH3T3 cells with v-Mos)	29 ± 11[a]	5	2	1.9	41
Ciras-1 (10T1/2 cells with H(T24)-Ras)	14 ± 5[a]	4	4	Not done	Not done
NR4 (10T1/2 cells with H(T24)-Ras)	2 ± 1.4[a]	3	3	Not done	Not done
NIH/hmyc1 (NIH3T3 cells with c-Myc)	1 ± 0.6[a]	1	1	3.5	24
10T1/2 (parental line)	0[a]	Not applicable[e]	1	(1)	16
NIH3T3 clone 7 (parental line)	0[a]	2	1	(1)	36
2H1 induced (NIH3T3 cells with MT-H(T24)-Ras, induced with Zn for 12 h	Not applicable	4	5	Not done	Not done
2H1 uninduced (NIH3T3 cells with MT-H(T24)-Ras, uninduced control)	Not applicable	1	1	Not done	Not done
ΔN3-S222D (NIH3T3 cells with constitutively active MAPKK)	Positive[b]	1	1	3.7	18
Wild-type Mapkk (control NIH3T3 cells with non-transforming wild-type MAPKK)	Negative[b]	1	3	(1)	Not done
K97 (NIH3T3 cells transfected with catalytically inactive MAPKK)	Negative[b]	1	1	0.6	19
Neo NIH3T3 (control for MAPKK transfections, transfected only with neomycin resistance)	Negative[b]	1	2	Not done	20

[a]Number of lung nodules ± standard deviation in standardized assay [Egan et al., 1987a,b].
[b]Ability to form subcutaneous tumors after local injection [Mansour et al., 1994].
[c]Chadee et al. [1995].
[d]Cytopathologist 1 declined judgment since the cells appeared different compared to MR4 cells.
[e]Cytopathologist 1 declined judgment due to the presence of rare three-dimensional clusters of cells with prominent nuclear membrane abnormalities.

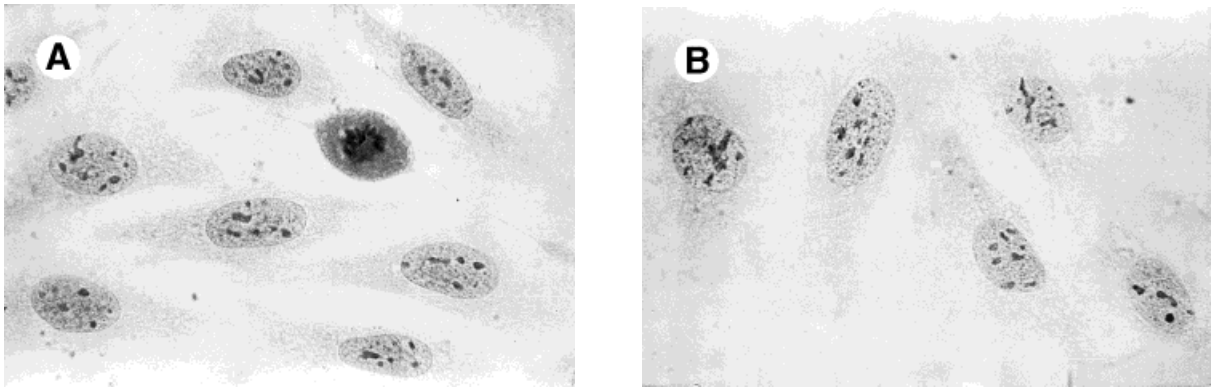


Fig. 3. Cells transformed with constitutively active MAPKK (ΔN3-S222D cells) (**A**) and cells transfected with catalytically inactive MAPKK (K97 cells) (**B**) both resemble the nontransfected parental cell lines. Papanicolaou stain, each ×1,000 original magnification.

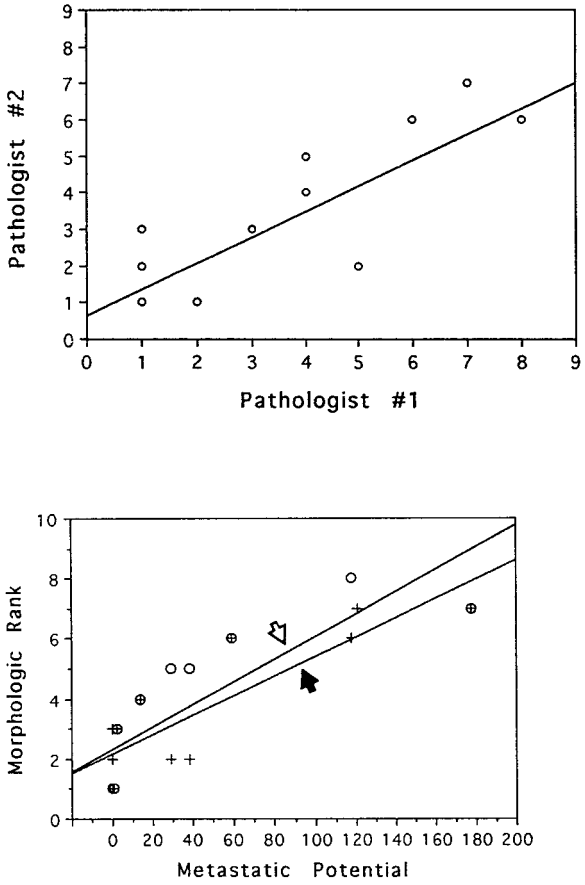


Fig. 4. **A:** Interobserver agreement between two cytopathologists in their rank order of increasing Ras-associated nuclear change. Correlation coefficient = 0.82, $P < 0.0002$ by f-test. **B:** Correlation of Ras-associated nuclear change and metastatic potential. The open arrow shows the simple regression line for pathologist 1 (open circles), with correlation coefficient = 0.83 and $P = 0.0014$ by f-test. The solid arrow indicates the simple regression line for pathologist 2 (crosses), with correlation coefficient = 0.86 and $P = 0.0003$.

ylation of one gene (ornithine decarboxylase) in NIH3T3 cells transformed by H-Ras.

Since the ΔN3-S222D-NIH3T3 cell line (bearing a constitutively active MAPKK, 400 times more active than wild-type MAPKK in activating MAPK [Mansour et al., 1994]) showed no nuclear change, the multiple nuclear transcription factor targets of MAPK per se would not appear to mediate the chromatin coarsening. While actin cytoplasmic filaments could conceivably help to mediate the observed changes in nuclear contour, there is no mechanism to explain how the known cytoskeletal components altered by RTK pathways could mediate the increased coarseness of the chromatin.

The observed nuclear morphologic changes may be related to alterations of the nuclear matrix—the protein/RNA filamentous structure that remains after extracting histones and digesting DNA from nuclei [Berezney et al., 1995; Fey and Penman, 1984]; the appearance of the nuclear matrix retains some of the landmarks of intact nuclei. The nuclear matrix consists of both filamentous proteins and globular proteins involved in replication and transcription. Since there are specific alterations of nuclear matrix proteins in specific forms of cancer [Bidwell et al., 1994; Khanuja et al., 1993] and since cancer cell nuclei typically show morphologic abnormalities, nuclear matrix proteins could be related to various diagnostic alterations in the appearance (or large-scale structural organization) of nuclei. A recent study of five of the same cell lines reported on in the present article has shown a correlation between the amount of a group of nuclear matrix proteins and metastatic potential as well as the

rank order of increasing Ras-associated nuclear change [Samuel et al., 1997]. The specific identities of these altered nuclear matrix proteins are presently unknown.

Mello and Chambers [1994] studied the relation of metastatic ability to large-scale chromatin structure (as assessed by image analysis of feulgen-stained cells) in a series of NIH3T3 cells transfected with H(T24)-Ras. They noted a phenotype of extreme contrast between condensed and noncondensed chromatin that appeared to correlate with metastatic ability but was difficult to quantify by image analysis texture parameters.

MAPKK expression in transfected NIH3T3 cells has been reported to lead to development of tumorigenicity as well as morphologic evidence of transformation [Cowley et al., 1994; Mansour et al., 1994]. The reported morphologic change consists of multilayering of the transfected cells in culture, growth in soft agar, rounding of cell shape, and refractivity of the cells by phase contrast microscopy. Cowley et al. [1994] showed a change from a flattened to a rounded cytoplasmic contour in low magnification actin-stained fluorescent micrographs of MAPKK-expressing cell lines. Further, they show that transfection with a dominant negative mutant of MAPKK reverts the rounded cytoplasmic morphology of Ras-transfected cells towards a flattened cytoplasmic morphology. Chromatin features are not described in either of these studies, however, and Cowley et al. [1994] found that injected anti-Ras antibodies cause a partial reversion of the rounded morphology of the constitutively active MAPKK transfectants, suggesting partial activation of a Ras pathway by their MAPKK mutant construct.

It is interesting that the Ras-associated nuclear change seems unrelated to growth rate. As an apparent corollary, the distalmost components of the mitogen signal transduction pathway per se do not transduce large scale nuclear structural changes. Further characterization of the Ras-associated nuclear change may permit the signal for nuclear morphologic changes to be followed.

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