The Conversion of Mouse Skin Squamous Cell Carcinomas to Spindle Cell Carcinomas Is a Recessive Event

A. B. Stoler,* F. Stenback,‡ and A. Balmain*

*Cancer Research Campaign Beatson Laboratories, Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD; and ‡Department of Pathology, University of Oulu, Oulu, Finland

Abstract. Squamous carcinomas of both human and rodent origin can undergo a transition to a more invasive, metastatic phenotype involving reorganization of the cytoskeleton, loss of cell adhesion molecules such as E-cadherin and acquisition of a fibroblastoid or spindle cell morphology. We have developed a series of cell lines from mouse skin tumors which represent different stages of carcinogenesis, including benign papillomas, and clonally related squamous and spindle carcinomas derived from the same primary tumor. Some spindle cells continue to express keratins, but with a poorly organized keratin filament network, whereas in others no keratin expression is detectable. All of the spindle cells lack expression of the cell adhesion molecule E-cadherin and the desmosomal component desmoplakin. Loss of these cell surface proteins therefore appears to precede the destabilization of the keratin network. At the genetic level, it is not known whether such changes involve activation of dominantly acting oncogenes or loss of a suppressor function which controls epithelial differentiation. To examine this question, we have carried out a series of fusion experiments between a highly malignant mouse skin spindle cell carcinoma and cell lines derived from premalignant or malignant mouse skin tumors, including both squamous and spindle carcinoma variants. The results show that the spindle cell phenotype as determined by cell morphology and lack of expression of keratin, E-cadherin, and desmoplakin proteins, is recessive in all hybrids with squamous cells. The hybrids expressed all of these differentiation markers, and showed suppression of tumorigenicity to a variable level dependent upon the tumorigenic properties of the less malignant fusion partner. Our results suggest that acquisition of the spindle cell phenotype involves functional loss of a gene(s) which controls epithelial differentiation.

THE progressive loss of the capacity to differentiate is a common feature of tumor development in both humans and animals. The metaplastic changes seen in certain early tumor types can be followed by the acquisition of a more undifferentiated or anaplastic phenotype characterized by the inability to produce the characteristic differentiation products of the tissue of origin, grossly altered morphology, and more aggressive tumorigenic properties. Such changes have been observed in tumors arising from a variety of body sites including the brain, skin, colon, and breast (Willis, 1973; Turusov, 1979; Guldberg, 1923).

The genetic basis for these progressive alterations is presently unknown, but is generally thought to involve mutations in proto-oncogenes or tumor suppressor genes (for reviews see Fearon and Vogelstein, 1990; Lasko et al., 1991). In human colon tumors, alterations at loci on chromosomes 5, 18, and 17 have been associated with more advanced histological grade. Genes mapping to these loci have recently been identified and shown to be mutated in colorectal tumors (Fearon et al., 1990; Nigro et al., 1989; Kinzler et al., 1991; Groden et al., 1991). Tumors of glial origin exhibit sequential losses of heterozygosity at different chromosomal loci during progression to anaplastic astrocytomas (Mikkelsen et al., 1991). The isolation and identification of the critical genes which are altered in these tumors would provide important information not only on the mechanisms of tumor progression, but on the genetic basis of differentiation control in many tissues.

One of the best characterized animal models for studying the genetic and biological alterations in tumor initiation and progression is the mouse skin system (Hecker et al., 1982). Multiple benign tumors develop on the dorsal skins of mice initiated by a single treatment with a chemical carcinogen, followed by twice weekly applications of a tumor promoter such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA). A proportion of these tumors progress to form squamous cell carcinomas which can be classified into various grades of malignancy according to histological criteria (Klein-Szanto, 1989). In extreme cases, spindle cell tumors are formed which display a highly aggressive, invasive phenotype (Klein-Szanto et al., 1989; Buchmann et al., 1991) associated with an altered cytoskeleton and lack of expression of cell adhesion molecules of the cadherin family (Diaz-Guerra et al., 1992; Navarro et al., 1991). In some spindle

cell lines, the keratin filament network appeared to be completely absent, as were E- and P-cadherin cell adhesion molecules, suggesting that in these cells the major characteristic gene expression pattern of epidermal differentiation has been switched off. Whether such changes occur in one or in several successive stages is however not known.

Some of the genetic changes associated with particular stages of carcinogenesis in this system have already been elucidated (for reviews see Balmain and Brown, 1988; Balmain et al., 1992). Initiation can take place by induction of Harvey-*ras* (H-*ras*) gene mutations, which occur in a carcinogen-specific manner (Quintanilla et al., 1986; Bizub et al., 1986; Brown et al., 1990). Further changes at the H-*ras* locus on mouse chromosome 7, including overrepresentation of the mutant allele and/or loss of the normal allele, are involved in tumor progression (Quintanilla et al., 1986; Bremner and Balmain, 1990; Bianchi et al., 1990). Studies on tumors induced in hybrid mice which display multiple restriction fragment length polymorphisms (RFLPs) have shown that loss of heterozygosity at alleles on chromosome 11, including the p53 tumor suppressor gene, is seen in ~30% of carcinomas, but not in papillomas (Bremner and Balmain, 1990; Burns et al., 1991; Ruggeri et al., 1991). Such tumors exhibit homozygous mutations in the remaining p53 allele.

The genetic basis of the transition to anaplastic spindle cell carcinomas is presently unclear. The use of clonal cell lines of either squamous or spindle morphology derived from the same tumor has enabled us to show conclusively that the p53 mutations are not responsible for progression to spindle cell phenotype. Cells of both squamous and spindle morphology exhibited the same set of mutations in the p53 alleles (Burns et al., 1991) but could be distinguished on the basis of an altered ratio of mutant to normal H-*ras* genes on chromosome 7 (R. Crombie, C. J. Kemp, S. Haddow, and A. Balmain, unpublished results). This result on clonal cell lines is entirely in agreement with experiments on primary tumors which demonstrated an imbalance of H-*ras* alleles in high grade or spindle cell carcinomas (Buchmann et al., 1991). The information available to date therefore suggests that the spindle cell phenotype is associated with imbalance of alleles on chromosome 7, but the nature of the critical target gene(s) remains unresolved. In this regard, it is of interest to note that the region of mouse chromosome 7 which harbors the H-*ras* locus shares synteny with loci on human chromosome 11 which can be either amplified (11q13) (Berenson et al., 1989) or show loss of heterozygosity (11p15) (Lasko et al., 1991) in human tumors. Among the possible explanations which are compatible with the data on mouse skin are therefore that progression is caused by increased activity of a dominantly acting oncogene or by loss of a suppressor function which controls the differentiated phenotype.

We have carried out immunofluorescence studies to analyze the expression patterns of keratins, E-cadherin and desmoplakin in a series of cell lines derived from benign and malignant skin tumors. The results suggest that loss of cell adhesion proteins may precede the destabilization of the keratins. Furthermore, the results of cell fusions between a spindle cell carcinoma and squamous cells derived from tumors at different stages of malignancy, show that development of the spindle cell phenotype is indeed a recessive event caused by loss of a gene(s) controlling the expression of a number of epidermal differentiation markers.

Materials and Methods

Cell Cultures

All of the cell lines were grown at 37°C in special liquid medium (SLM; Flow Laboratories, Research Triangle Park, NC) supplemented with 10% FBS and 4 mM glutamine (GIBCO BRL, Gaithersburg, MD) in an incubator adjusted to 5% CO_2, except MSC P1(P1) and MSC P6(P6) which were grown in SLM supplemented with 5% FBS and 400 ng/ml cholera toxin on a feeder layer of NIH 3T3 cells. These cells were made feeder independent 48 h before fusion by growth in KGM media with a Ca^{2+} concentration of 0.05 mM and addition of EGF, insulin, hydrocortisone, bovine pituitary extract, and antibiotics according to the manufacturer (Clonetics Corporation, San Diego, CA). The origin of the cell lines used has been described previously (Diaz-Guerra et al., 1992; Burns et al., 1991; Quintanilla et al., 1991; Haddow et al., 1991).

The carB 6-thioguanine- and neomycin-resistant clone used for fusions was generated by initial selection in 100 μM 6-thioguanine. After this selection, cells were infected using polybrene with the retrovirus pZipNeoSV(X) to make them neomycin resistant (Cepko et al., 1984). Several 6-thioguanine- and neomycin-resistant clones were isolated and one clone, carB-5A was used in fusions.

Cell cultures were routinely monitored for mycoplasma infection and were found negative.

Cell Fusions

Cells were harvested, washed with PBS to remove residual Ca^{2+}, then cocultured in 60-mm dishes at a 1:5 ratio (carB-5A always in lower concentration) in 0.05 mM Ca^{2+} KGM media plus additives for 24 h. The monolayer of cells was then fused by a 75-s exposure to 50% wt/vol polyethylene glycol 1500 (PEG; Boehringer Mannheim, GmbH, Mannheim, Germany). The PEG was immediately removed from plates, which were washed three times with calcium-free medium, followed by fresh medium containing calcium for 1 h, then refed and incubated overnight at 37°C. The following day, cells were harvested and assayed for hybrid formation by plating between 1 and 10 × 10^4 cells per 100-mm dish in hypoxanthine/aminopterin/thymidine (HAT, GIBCO BRL) plus 0.3 or 0.4 mg/ml geneticin (G 418, GIBCO BRL) in special liquid media containing 10% FCS. Liquid platings were refed twice weekly and counted after 2–4 wk. Since the parental carB-5A cells are resistant to both 6-thioguanine and neomycin, they can be used as a "universal donor" in cell fusion experiments. Parental carB-5A cells die when cultured in HAT medium, whereas the other parental fusion partner is killed in the presence of G418. Only genuine hybrid cells should survive the double selection procedure.

Hybrid Analysis

At least 10 ring clone colonies isolated from separate dishes for each cross were picked from HAT/G418 liquid plates and expanded. Three or more separate fusion events were performed for each cross. Hybrids were maintained in selective media, expanded, then used for isolation of RNA, DNA, immunocytochemistry, or injection into nude mice. Care was taken to use the same passage cells for each separate analysis. The experiments described below were carried out on at least two, and in some cases four, independent hybrid clones of each type, with the exception of carB × carB, where in general only one clone was used.

DNA and RNA Analysis

High molecular weight genomic DNA was extracted by lysing either parental or hybrid cells in T25 flasks with lysis buffer containing 50 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl, 5mM DTT, 1% SDS, and 0.5 mM spermidine. Proteinase K was added to a final concentration of 1 mg/ml, then the solution was incubated for 24–36 h at 37°C. This was then extracted with phenol-chloroform, precipitated, and the pellet was air dried and resuspended in tris-EDTA at 500–1,000 μg/ml.

Total RNA was extracted using RNazol B (Cinna/Biotex) according to manufacturer's recommendations. Northern blotting was carried out, using 20 μg of total RNA run on 1% agarose gels and transferred to Hybond-N membranes (Amersham International, Buckinghamshire, England) under conditions recommended by the supplier. A probe for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene was generated by PCR amplification of a cDNA fragment based on the published rat sequence (Fort et al., 1985). A mouse E-cadherin cDNA probe was kindly provided by M. Takeichi. Probes were labeled by the random priming procedure (Feinberg and Vogelstein, 1983).

RNase Protection Assay

An anti-sense murine H-*ras* exon 1-2 was used in an RNase protection assay (Sambrook et al., 1989). Briefly, anti-sense murine H-*ras* exons 1-2 from murine *ras* nucleotide 1384 to 1980 (Brown et al., 1988), was labeled using a SP6/T7 transcription kit (Boehringer Mannheim) and 100 μCi of ^{32}PCTP to generate a 600-bp fragment. 20 μg of either parental or hybrid RNA was incubated with labeled *ras* antisense probe in a hybridization mix at 56°C overnight. 40 μg/ml of RNaseA and 150 μg/ml of RNase T_1 was added to the hybridization mix for 1 h at 30°C. Proteinase K and SDS was added for 15 min to stop the reaction, then adding yeast tRNA as a carrier, the solution was extracted with phenol-chloroform, washed, precipitated, and resuspended in 5 μl of loading buffer. All of the sample was run on an 8% denaturing acrylamide sequencing gel for 2 h at 50 W. The gel was removed, dried, and exposed to autoradiographic film. The normal *ras* band is 179 nucleotides (nt)[1], the upper mutant *ras* band is 110 nt and the lower mutant *ras* band is 69 nt.

Immunofluorescence

Cells were grown on 16-mm washed coverslips placed in 60-mm dishes until 80% confluent. Cells were washed in PBS containing 1 mM $CaCl_2$, fixed for 30 min in ethanol at −20°C, then air dried. For keratin localization, an alternative fixation can be used. Coverslips were fixed 30 min in 10% formalin, then 5 min in −20°C acetone and 3 min in −20°C methanol (Sun and Green, 1978). Coverslips were incubated first with a 5% powdered milk solution, then with either a monoclonal pan keratin antibody (Lu 5. Boehringer Mannheim) at 1 mg/ml or a polyclonal rat E-cadherin antibody, ECCD-2 (a generous gift from M. Takeichi) (Yoshida-Noro et al., 1984), diluted 1:500 in PBS containing 1 mM $CaCl_2$. Cells were also stained with the antibody DP1 and 2 - 2.15 which recognizes the desmosomal proteins desmoplakin I and II (Cowin et al, 1985*a*). The antibody was used as a 1:1 dilution in the same buffer. After incubation for 1.5 h., PBS containing 1 mM $CaCl_2$ was used for washing. For visualization, fluorescein-conjugated secondary antibody was used either to rat (Sigma Immunochemicals, St. Louis, MO; 1:20) or mouse (Vector Laboratories, Inc., Burlingame, CA; 1:75), according to manufacturer's recommendations. A Leitz fluorescent microscope was used to visualize samples. Two different hybrid clones of each kind were used in immunofluorescence studies, with the exceptions of carB fused with itself, where one clone was used, carB × P6 (three clones) and carB × P1 (four clones).

Tumorigenicity Assay

Parental and hybrid cells were harvested, washed, resuspended in PBS, then 1×10^6 cells were injected subcutaneously at two sites in the abdominal region of male CBA/nu mice. Tumor growth was measured 2-3 times weekly until tumors reached ~1.0 cm in diameter, at which time the animals were sacrificed. Tumorigenicity was tested for four independent hybrid clones of each type.

Histology

Tissues for histological examination were fixed in 4% buffered formalin overnight, dehydrated and embedded in paraffin by standard methods. Sections (5 μm) were stained with hematoxylin and eosin.

Results

Progressive Loss of Epidermal Differentiation Markers in Spindle Carcinomas

A series of cell lines representative of different stages of skin carcinogenesis were studied for the expression of three epidermal differentiation markers–keratins, the E-cadherin cell adhesion molecule, and desmoplakin. Most of the cell lines used have been described previously (Diaz-Guerra et al., 1992; Burns et al., 1991; Quintanilla et al., 1991). C5N is a nontumorigenic, immortalized cell line isolated by single cell cloning of MCA3D cells derived by Kulesz-Martin et al. (1983) from a Balb/c mouse. MSC P6 (P6) and MSC P1 (P1) are papilloma lines isolated from (Dimethylbenzanthracene) DMBA/TPA treated *spretus* ×CBA F_1 mice (Haddow et al., 1991). B9 is a squamous cell carcinoma from a multiple DMBA-treated *spretus* × CBA F_1 hybrid mouse and A5 is a spindle cell carcinoma variant isolated from the same tumor (Burns et al., 1991). CarB is a highly aggressive spindle cell carcinoma isolated from a NIH mouse after DMBA/TPA treatment. An important criterion for choosing these cell lines is that all except C5N were obtained from tumors initiated in vivo with DMBA, and consequently carry the H-*ras* mutation at codon 61 which is typically induced by this initiating agent (Quintanilla et al., 1986, and data not shown).

The in vitro morphological characteristics of these isolated cell lines are shown in Fig. 1. C5N, P1, P6, and B9 (*A*, *C*, *D*, and *E*) have a typical epithelioid morphology, being cuboidal in shape, with a cobblestone pattern of growth. Under appropriate conditions, these cells are able to differentiate in culture, they enlarge, flatten, and eventually stratify. A5 and carB-5A, *F* and *B*, respectively, have a spindle or fibroblastoid morphology, and give rise to spindle cell carcinomas upon injection into nude mice (Diaz-Guerra et al., 1992; Burns et al., 1991).

A major characteristic of epidermal cells is the ability to express keratins, a family of intermediate filament proteins consisting of at least 20 members (for reviews, see Fuchs, 1988; Moll et al., 1982). Members of this family can be expressed in a tissue-specific or differentiation stage-specific manner, as well as at various times during development (Kopan and Fuchs, 1989). In addition, the pattern of keratin expression can change upon malignant transformation. Several groups have demonstrated the expression of ectopic keratins in epidermal cells transformed by *ras* oncogenes (Nischt et al., 1988; Diaz-Guerra et al., 1992; Cheng et al., 1990) or by DNA tumor viruses (Hronis et al., 1984). Therefore, a broad spectrum keratin antiserum was used to investigate the general keratin expression in these cells (Fig. 2). C5N, P1, P6, and B9, *A*, *C*, *D*, and *E*, respectively, have a typical pattern of keratin expression: a filamentous network that traverses the cells to the periphery. Interestingly, the spindle cell carcinoma A5 (*F*) displays a keratin network, but with aberrant filaments in some cells (*inset*). In agreement with previous results (Diaz-Guerra et al., 1992), carB-5A, the other spindle cell carcinoma, does not express any keratin protein, but does express vimentin (data not shown).

E-cadherin (or uvomorulin) is a member of the calcium-dependent family of cell adhesion proteins (Takeichi, 1991; Kemler and Ozawa, 1989). These molecules play a pivotal role in cell–cell interaction, exhibit a unique spatiotemporal expression pattern during embryogenesis and seem essential for normal morphogenesis (Gumbiner, 1992). Different cell types express a different set of cadherins and binding is via recognition to identical molecules on adjacent cells. E-cadherin is exclusively found on epithelial cells (Yoshida-Noro et al., 1984). Although there is a strong amino acid homology between family members (Hirai et al., 1989; Nose et al., 1987), individual members can be distinguished immunologically (Hirai et al., 1989). To examine E-cadherin expression in parental cell lines, immunofluorescence was carried out using a mAb to E-cadherin, ECCD-2 (Yoshida-Noro et al., 1984; Nose et al., 1987). In Fig. 3, C5N, P1, P6, and B9 (*A*, *C*, *D*, and *E*) demonstrate typical E-cadherin staining, showing strong fluorescence at cell–cell contact regions. P6 (Fig. 3 *C*) has staining in a wave-like pattern which can

1. *Abbreviation used in this paper*: nt, nucleotide.

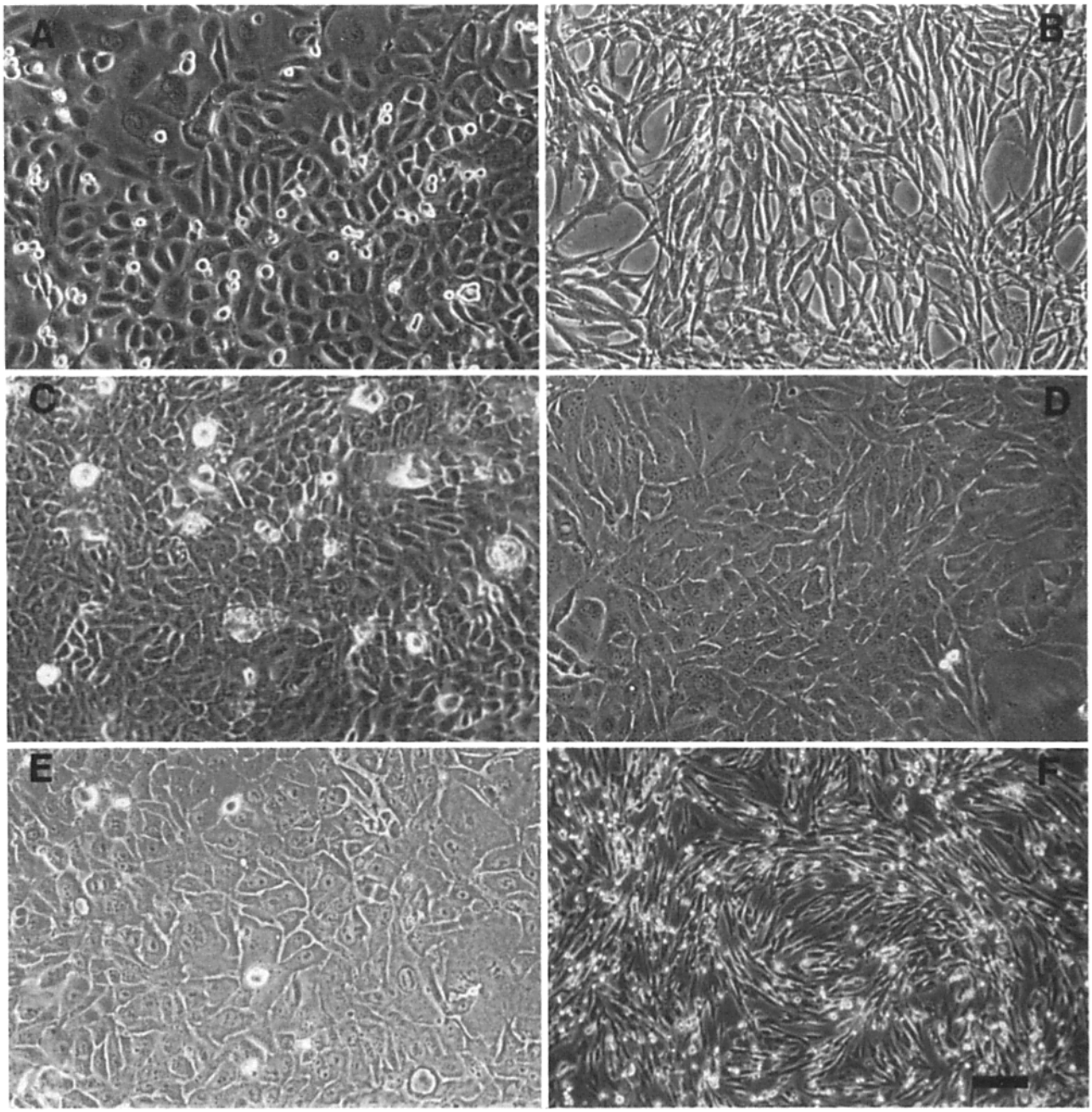

Figure 1. Phase-contrast micrographs of the morphology of parental mouse cell lines used in fusions. (*A*) C5N, an immortalized mouse keratinocyte line; (*B*) carB–5A, a spindle cell carcinoma line; (*C*) P6, and (*D*) P1, two papilloma cell lines isolated from separate papillomas; (*E*) B9, a squamous cell carcinoma; and (*F*) A5, a spindle cell carcinoma cell line. B9 and A5 were isolated from the same tumor. *A*, *C*, *D*, and *E* have a typical epithelioid morphology whereas *B* and *F* have a fibroblastoid or spindle morphology. Bar, 330 μM.

be attributed to overlapping cell membranes. Staining is specific for contact regions as individual cells or isolated colony borders are unstained (data not shown). In contrast to this pattern of staining seen in cells with an epithelial morphology, the spindle cell carcinomas carB-5A and A5 (Fig. 3, *B* and *F*) are negative for E-cadherin expression. This result confirms and extends a previous observation that carB fails to express E-cadherin (Navarro et al., 1991).

Desmosomes are important structures which are involved in the maintenance of cell–cell adhesion and mechanical stability in a variety of epithelial cell types (for review see Cowin et al., 1985*b*). They are intimately associated with the cytoskeleton and are downregulated at the transition from epithelial to mesenchymal cells during embryonic development (Franke et al., 1982) or upon treatment of certain carcinoma cells with agents which induce a similar transition in vitro (Boyer et al., 1989). There is also some evidence for an alteration in the polarized localization of desmosomal proteins in undifferentiated adenocarcinomas of the colon (Collins et al., 1990). It was therefore of interest to determine whether desmosomal proteins show an altered pattern of expression in tumor cells at different stages of carcinogenesis.

All of the cell lines used in this study were stained with the antibody DP1 and 2, which recognizes the desmosomal proteins desmoplakin I and II (Cowin et al., 1985*a*). The

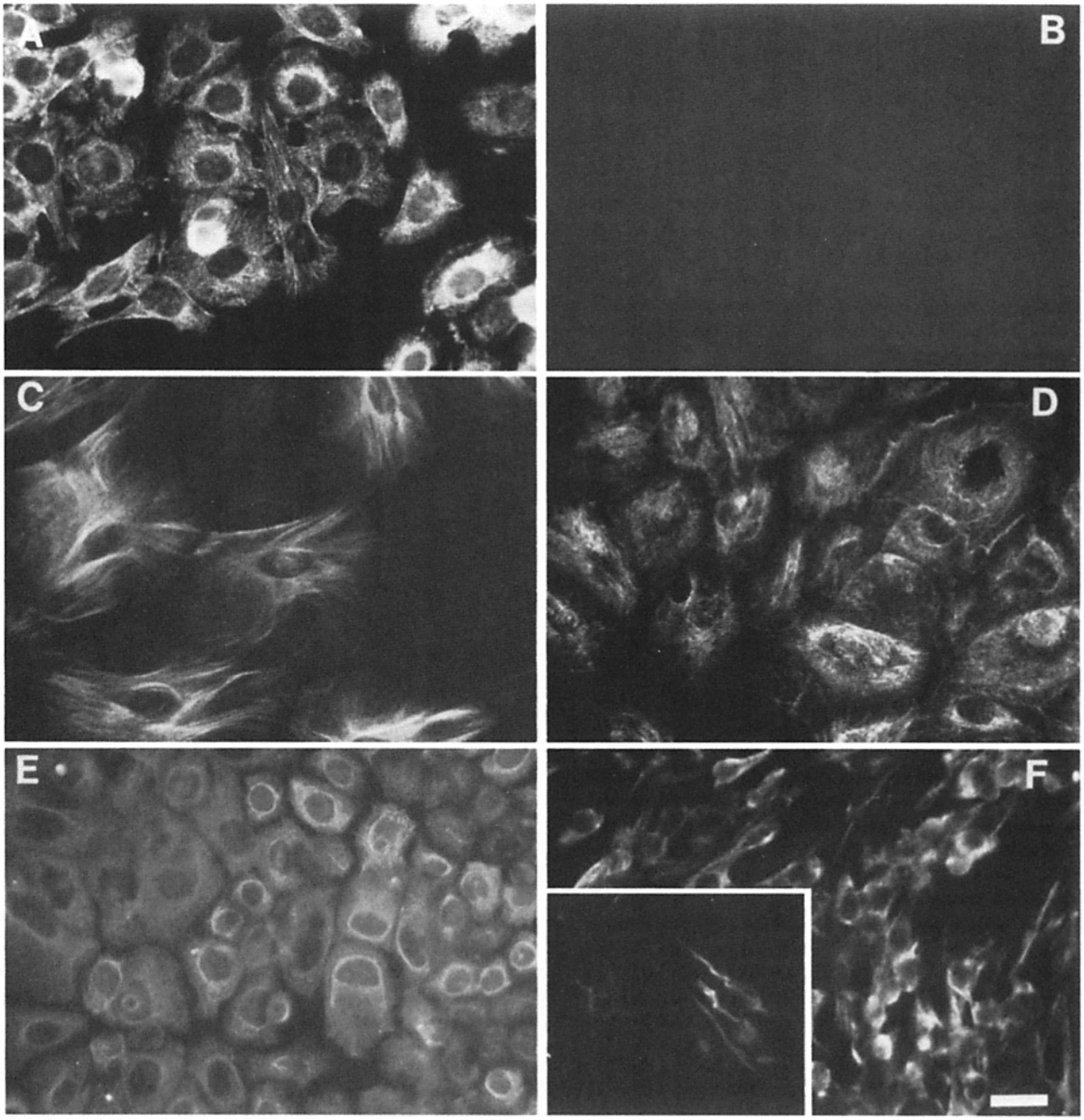

Figure 2. Immunofluorescent localization of keratin in parental cell lines. Cells were fixed in either −20°C ethanol or 10% formalin, then exposed to a pan-keratin mouse mAb. Bound antibodies were then visualized by indirect, immunofluorescence using an FITC-conjugated secondary antibody. (*A*) C5N; (*B*) carB-5A; (*C*) P6; (*D*) P1; (*E*) B9, and (*F*) A5. *A*, *C*, *D*, and *E* have an extensive keratin network, characteristic of epithelial cells. The spindle cell carcinoma carB-5A (*B*) is negative for keratin. A5 (*F*), a different spindle cell carcinoma line, contains a keratin network, but exhibits some abnormal filaments (*inset*). Bar, 25 μM.

papilloma cell lines P6 and P1 (Fig. 4, *B* and *C*) showed a uniform pattern in all cells of punctate staining along the cell boundaries. The squamous carcinoma line B9, on the other hand, showed a less regular pattern in which the membrane localized staining appeared restricted to the larger, differentiating cells. Fig. 4 *D* appears slightly out of focus to illustrate the membrane staining in the more suprabasal cells, whereas the underlying smaller cells show a reduced level of expression which is also more diffusely spread throughout the cell. B9 cells growing at low density showed predominantly the latter pattern of staining (data not shown). No specific staining was seen in either of the spindle cell lines carB-5A (Fig. 4 *E*) or A5 (not shown). Both the desmoplakin proteins I and II and the E-cadherin cell adhesion protein therefore are absent from the spindle cell carcinomas.

Loss or reduction of desmoplakin is not however necessarily associated with tumor progression. The immortalized keratinocyte cell line C5N, and the original MCA3D line from which it was derived by single cell cloning, showed islands of cells which stained positively with the anti-desmoplakin antibody, particularly at intercellular contract regions (Fig. 4 *A*) but many cells in the same cultures stained

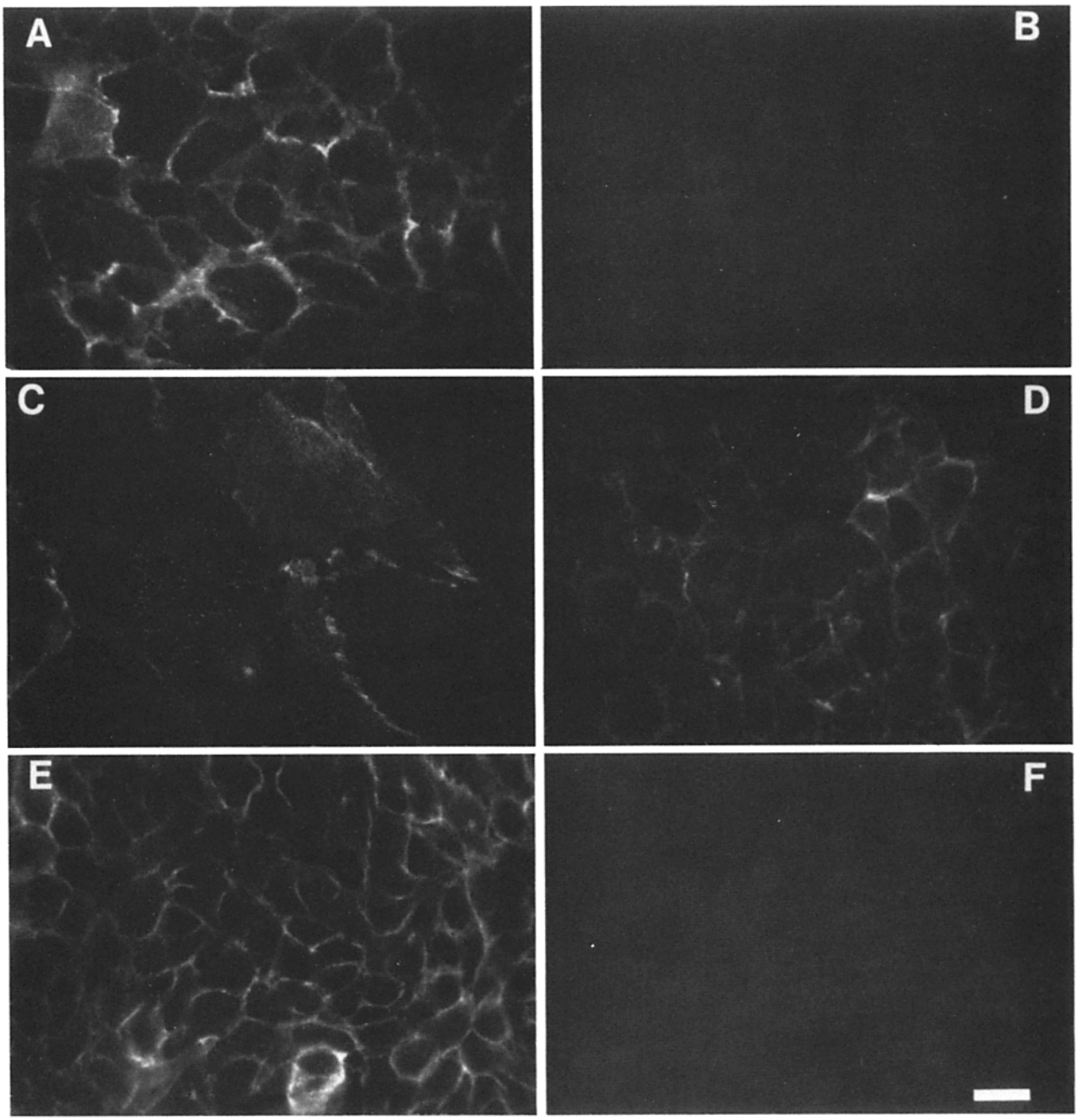

Figure 3. Immunofluorescent localization of E-cadherin in parental cell lines. Fixed cells were exposed to ECCD-2, a rat polyclonal antibody specific for E-cadherin. A FITC-conjugated secondary antibody was used to visualize bound antibody. (*A*) C5N; (*B*) carB-5A; (*C*) P6; (*D*) P1; (*E*) B9, and (*F*) A5. *A*, *C*, *D*, and *E* demonstrate E-cadherin localization at contact regions between cells. Both of the spindle cell carcinoma lines, carB -5A (*B*) and A5 (*F*), lack E-cadherin expression. Bar, 25 μM.

diffusely or not at all (Fig. 4 *D*, *arrows* indicate cells showing only background levels of staining). The expression pattern of E-cadherin in these cultures was however much more uniform (Fig. 3 *A*) indicating that desmoplakin and E-cadherin are not necessarily co-expressed in these keratinocyte-derived cells.

The Spindle Cell Phenotype Is Recessive in Cell Hybrids

The results outlined above show that progression to the spindle cell phenotype involves loss of expression of both keratins and cell adhesion molecules. Cell fusion is a well characterized approach to answer the question of whether a specific phenotypic trait, including malignancy, is dominant or recessive (for reviews see Stanbridge et al., 1982; Harris, 1988). In many cases, it has been shown that malignancy is recessive, and is suppressed in fusions between normal and tumor cells. Tumor suppression can be associated with the expression of differentiation markers in some hybrid cells (Peehl and Stanbridge, 1982; Harris and Bramwell, 1987), but not in others (Bader et al., 1991). Similar experiments have not to our knowledge been carried out with epidermal

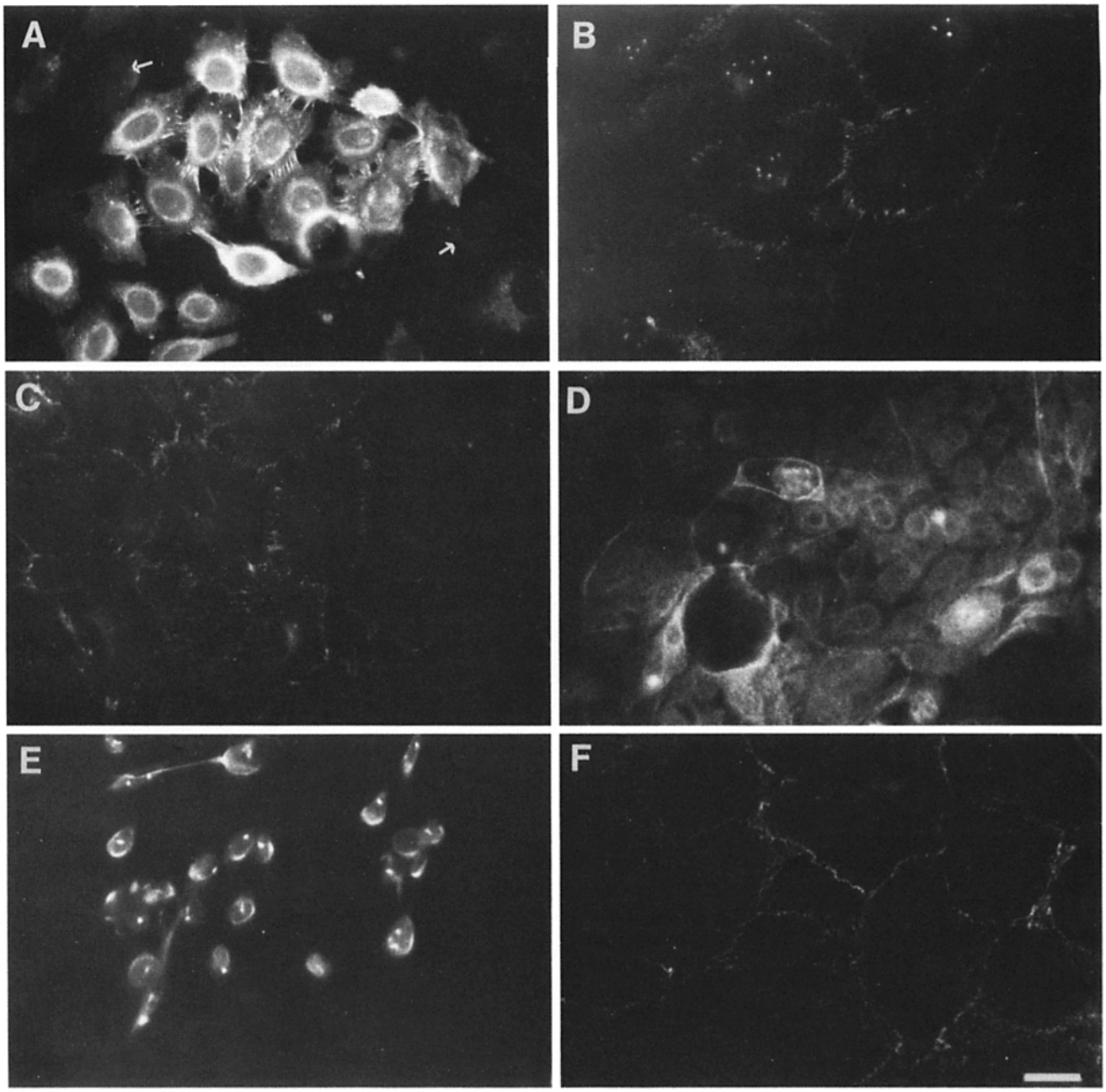

Figure 4. Immunofluorescent localization of desmoplakin in cell lines. Fixed cells were stained with antibody to desmoplakin as described in Materials and Methods. (*A*) MCA 3D cells; similar results were obtained with the single cell clone C5N; (*B*) P6; (*C*) P1; (*D*) B9; (*E*) carB-5A; and (*F*) hybrid between carB-5A and P6 cells. The arrows in *A* indicate cells which show only a background level of staining. The pattern seen in carB–5A cells (*E*) is nonspecific as shown by parallel staining without the primary antibody (not shown). Bar, 25 μM.

cells at various stages of tumorigenesis. Such studies could yield important information on the nature of progression from one stage of malignancy (squamous carcinoma) to another (spindle carcinoma) as well as on the degree of suppression seen in fusions between early and late tumor cells from the same lineage.

For this reason, cell fusions were carried out between carB, representing the most extreme end of the spectrum in terms of malignancy, and various cells at earlier stages. A 6-thioguanine, and neomycin, resistant clone carB–5A, with identical characteristics of morphology and tumorigenicity to the parental cell population, was used in the fusion experiments.

The results of this experiment are shown in Fig. 5. Virtually all of the hybrids generated between carB–5A and the immortalized keratinocyte line C5N had a clear epithelial morphology (Fig. 5 *A*). The cells grew as discrete epithelial islands which appeared to be stable over several passages in culture. This morphology could not be attributed to the fusion event itself, since fusions between carB–5A and either the parental carB cells (Fig. 5 *B*) or the other spindle cell line A5 (Fig. 5 *F*) both gave rise to hybrids with a spindle

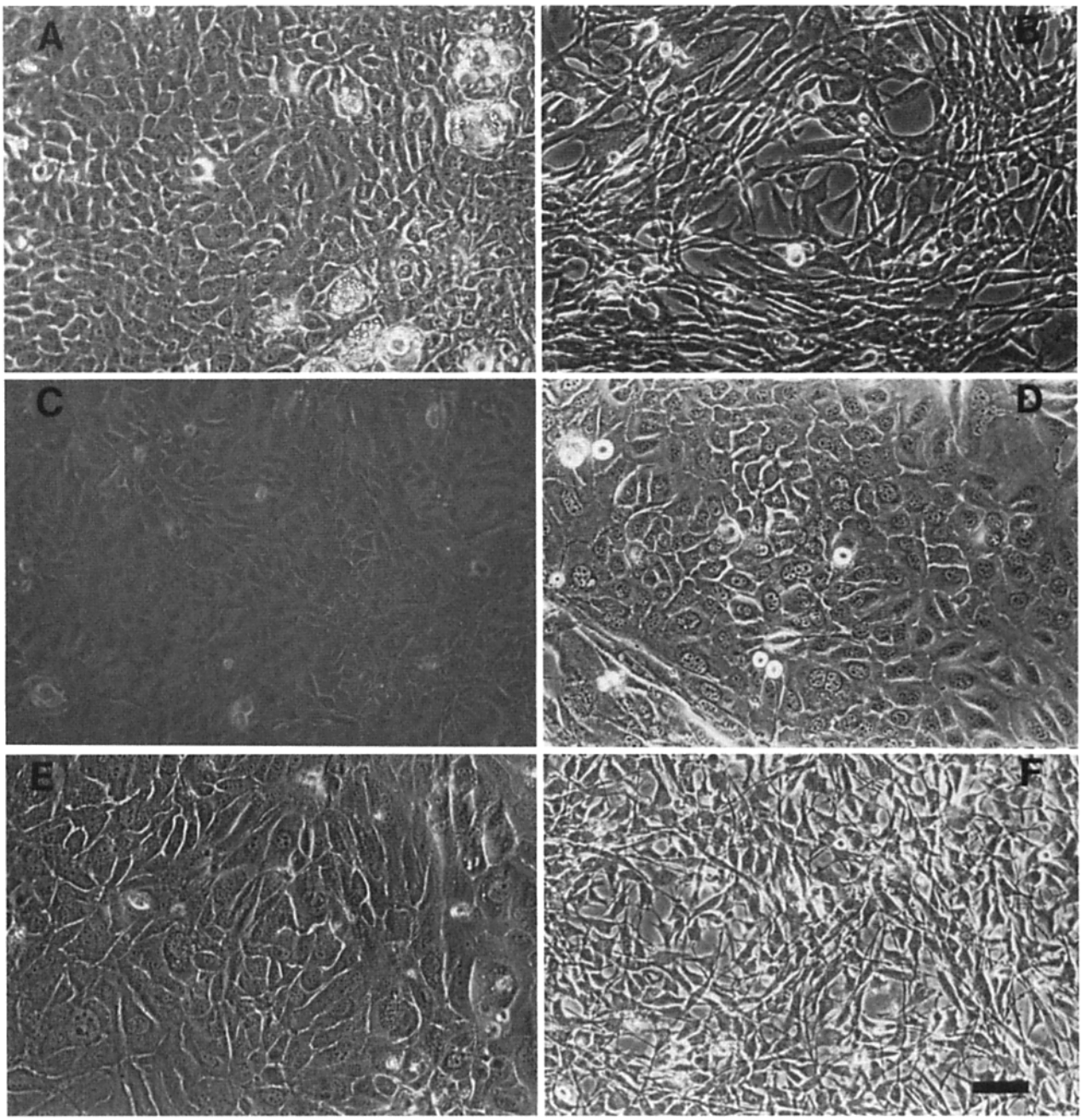

Figure 5. Phase-contrast micrographs of hybrid cell morphologies. Hybrids were generated by fusion of a genetically marked carB (carB–5A) to itself or one of the other parental cell lines as described in Materials and Methods. Shown is the morphology of a typical hybrid generated between carB–5A (*B*) and (*A*) the parental cell line C5N (B × C5N = hybrid); (*B*) the parental cell line carB (B × B); (*C*) the parental cell line P6 (B × P6); (*D*) the parental cell line P1 (B × P1); (*E*) the parental cell line B9 (B × B9) and (*F*) the parental cell line A5 (B × A5). Note that *A*, *C*, *D*, and *E* demonstrate that hybrids derived from fusions between an epithelioid and spindle cell have an epithelioid morphology whereas hybrids generated between two spindle cells (*B* and *F*) are spindle in morphology. Bar, 330 μM.

morphology. These results therefore demonstrated that the spindle cell phenotype is recessive, resulting from loss of information which determines cellular morphology. Moreover, since no complementation was observed in hybrids between carB–5A and A5, the underlying genetic defect is in a similar pathway in two independently arising spindle cell carcinomas.

A similar epithelial phenotype was seen in fusions between carB–5A and the other cell lines with epithelial morphology, including the two papilloma cell lines P6 and P1 (Fig. 5, *C* and *D*) and, importantly, the squamous carcinoma B9 (Fig. 5 *E*). This squamous carcinoma line is of the same clonal origin as the spindle cell line A5, since both lines were derived from the same primary tumor and they exhibit an identical series of mutations in the H-*ras* and p53 genes (Burns et al., 1991). The fact that B9 cells can suppress the spindle morphology whereas A5 cannot therefore localizes the complete loss of differentiation control to the transition between these two cell types.

Occasionally, some early hybrid clones between the papil-

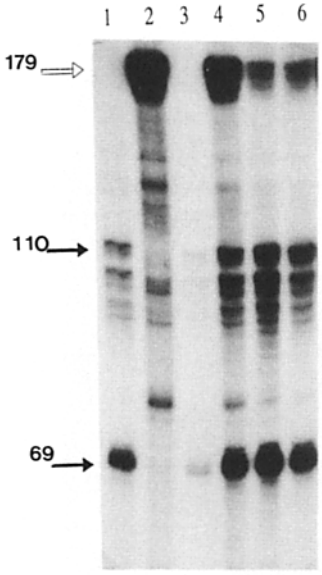

Figure 6. RNase protection of *ras* on mouse chromosome 7 in hybrids and parental cell line. RNase protection for *ras* was performed as in Materials and Methods. Lane *1*, carB-5A parent; lane *2*, C5N parent; lane *3*, B × B hybrid; and lane *4*, B × C5N hybrid; lane *5*, B × P6 hybrid; and lane *6*, B × P1 hybrid. The white arrow represents the presence of normal *ras* RNA (179 nt) and the black arrows represent the presence of mutant *ras* RNA (110 and 69 nt, respectively). CarB-5A parent has only mutant *ras*, C5N has only normal *ras* whereas the hybrids contain transcripts for both the normal and mutant *ras*.

loma line P1 and carB-5A appeared spindle shaped. However, inspection of the parental P1 cells showed the presence of both squamous epithelial cells and a sub-population of spindle shaped cells. We therefore assume that the spindle shaped hybrids arose by fusion between carB-5A and cells in this sub-population. It can therefore be concluded that fusions between a spindle carcinoma line and squamous cells at different stages of malignancy produce hybrids of epithelial morphology.

Hybrid Cells Contain both Parental Genomes

Cells were determined to be hybrids not only by growth in selective media which should kill any parental cells, but also by molecular analysis using microsatellite sequences. Microsatellite sequences are highly repetitive sequences, usually $(CA)_n$, randomly distributed in the mammalian genome (Cornall et al., 1991). These microsatellite sequences are highly polymorphic in length between human individuals and also between inbred mouse strains.

Since the parental carB-5A cells were derived from NIH mice, whereas the other cells were from *M. spretus*/CBA F_1 hybrids (B9, A5, P6, and P1), or from Balb/C (C5N), it was possible to verify the parentage of hybrids using polymorphic microsatellite sequences on mouse chromosomes 17, 18, and 11 (data not shown). The possibility of random chromosome loss cannot be eliminated, but these controls demonstrated that the hybrids are genuine, containing both sets of parental genomes.

Additional evidence for the hybrid nature of the fusion products comes from analysis of the expression of normal and mutant *ras* genes, by RNase protection (Fig. 6). CarB-5A has a homozygous mutation at codon 61 of the mouse H-*ras* gene, and does not contain the normal H-*ras* allele (Bremner and Balmain, 1990). A probe spanning exons 1 and 2 of the H-*ras* gene can be used to detect the expression of a mutant H-*ras* allele by RNase protection. The normal H-*ras* transcript gives rise to a protected fragment of 179 nt. (Fig. 6, *white arrow*) whereas mutation at codon 61 results in cleavage to give two bands at 110 and 69 nt. (*black arrows*). C5N cells show only the full size fragment corresponding to the normal mRNA (Fig. 6, lane *2*), and carB-5A has two fragments corresponding to the presence of only mutant mRNA (Fig. 6, lane *1*). However, hybrids between C5N and carB-5A cells show both normal and mutant-specific bands (Fig. 6, lane *4*). A band corresponding to expression of the normal H-*ras* gene is present in most of the carB-5A cell hybrids (Fig. 6, lanes *4*, *5*, and *6*). This band can only have come from the squamous cells used for fusions with carB-5A, and supports the conclusion that genuine hybrids have been formed.

Keratin Expression in Hybrid Cells

It was important to determine whether the typical differentiation markers of epidermal cells, the keratins, are expressed in hybrids with carB-5A cells which, as shown above, are completely lacking keratin proteins. Immunofluorescence was therefore carried out using a pan-keratin antibody (Fig. 7). Hybrids between carB-5A and C5N, P6, some clones of P1 and B9 all showed typical keratin expression, as seen in Fig. 7, *A*, *C*, *D*, and *E*. All are flat, cuboidal shaped cells with intact filament networks.

On the other hand, a different pattern was observed in fusions between the two spindle cell lines A5 and carB. Hybrids between carB-5A and itself lack keratin staining (Fig. 7 *B*) similar to the parental cell line. However, hybrids between carB-5A and A5 show punctate staining for keratins, in some cases perinuclear, while in other cells the staining is seen throughout the cytoplasm (Fig. 7 *F*). This is clearly different from the parental A5 line which still has a filamentous network (Fig. 2 *F*). It therefore appears that the fusion event has caused collapse of the keratin network. The presence of positively staining aggregates demonstrates that some keratin protein is being synthesized, but is for some reason unable to form stable networks. These results demonstrate that the B × A5 hybrids are intermediate between the two parental cells in terms of keratin expression. A5 parental cells do express keratins, but the filaments are aberrant, whereas carB-5A shows no keratin expression. Further studies of the nature of the keratins in these hybrid cells is in progress.

E-cadherin and Desmoplakin Expression Correlates with Cellular Morphology

Hybrids between carB-5A and C5N, P6, P1, and B9 all showed E-cadherin expression similar to the corresponding epithelial parental lines shown in Fig. 3, with staining localizing to contact regions between cells (data not shown). Hybrids between the two spindle cell lines demonstrated negative staining for E-cadherin (data not shown).

The E-cadherin expression detected by immunofluorescence correlated with the results of Northern analysis using a cDNA probe for E-cadherin (Fig. 8). CarB-5A was completely negative for E-cadherin mRNA (Navarro et al., 1991; Fig. 8, lane *2*) as were the homologous hybrids between carB-5A and the parental car-B cells (Fig. 8, lane *5*). C5N cells expressed E-cadherin mRNA (Fig. 8, lane *3*) at a similar level to B × C5N hybrids (Fig. 8, lane *1*) or B × P1 hybrids (Fig. 8, lane *4*). The other cell lines and hybrids analyzed led to the same conclusion: cell lines and hybrids of epithelial morphology expressed E-cadherin mRNA but spindle cells did not (data not shown). These results show that control of E-cadherin gene expression in these cells is transcriptional or posttranscriptional (e.g., mRNA stability) rather than at the level of translation.

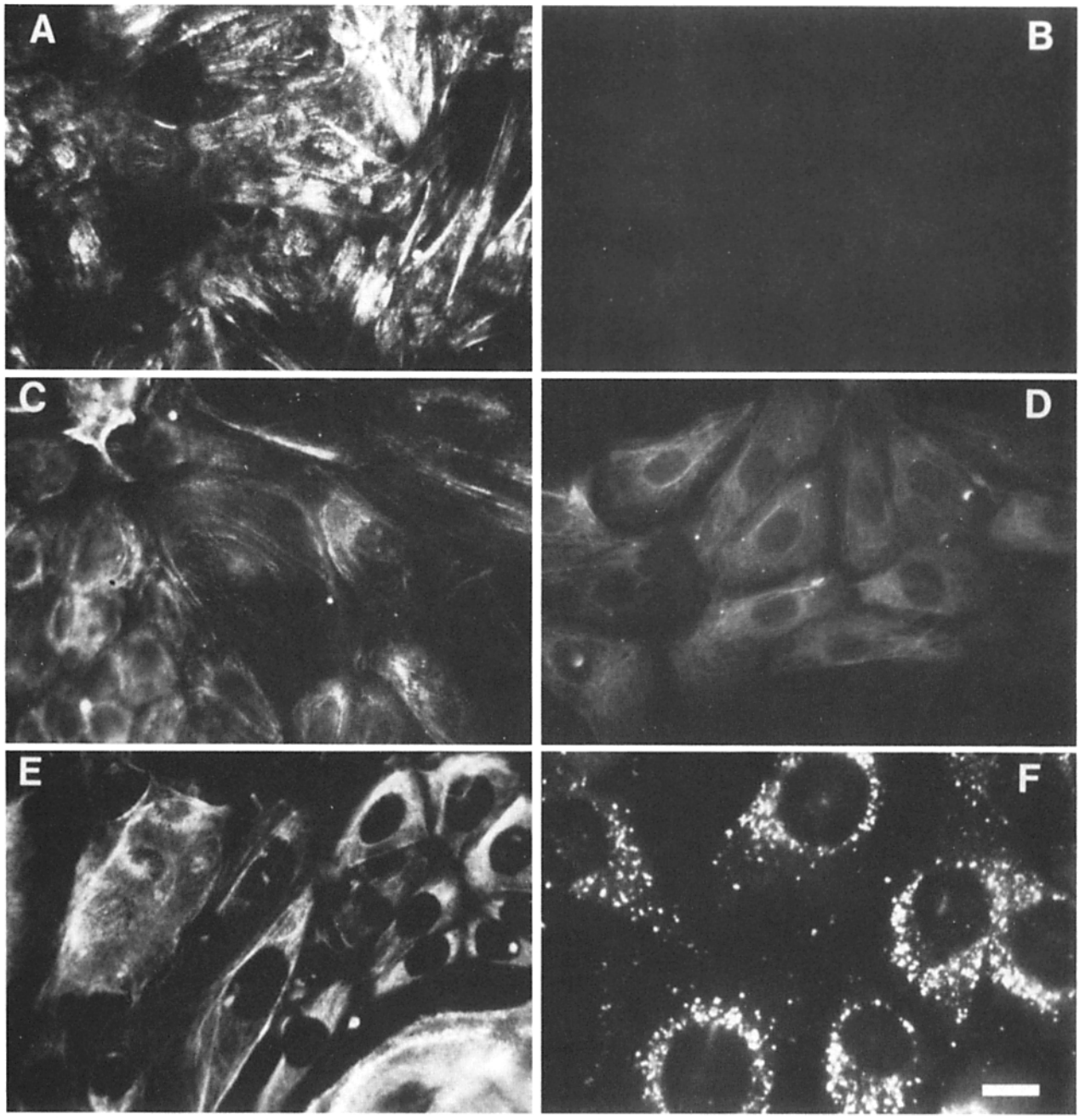

Figure 7. Immunofluorescent localization of keratin in hybrid cells. A pan-keratin mouse mAb was used to localize keratin filaments in hybrid cells. A FITC-conjugated secondary antibody was used to visualize bound antibody. (*A*) B × C5N; (*B*) B × B; (*C*) B × P6; (*D*) B × P1; (*E*) B × B9; and (*F*) B × A5. Hybrids with an epithelial morphology (*A*, *C*, *D*, and *E*) have a keratin network. The spindle hybrid B × B (*B*) was negative for keratin whereas the hybrid B × A5 (*F*) showed positive, but punctate, localization for keratin. Bar, 25 μM.

All of the cell hybrids analyzed for E-cadherin expression were also stained with the antidesmoplakin antibody. For example, hybrids between carB-5A and the papilloma line P6 showed membrane-localized expression of desmoplakin apparently identical to that of the P6 line itself (Fig. 4 *F*). Hybrids with the other cell lines gave similar patterns of staining to those in the corresponding squamous parental cells (data not shown).

Tumorigenicity of Hybrid Cells Is Suppressed to the Level of the More Normal Parent

Previous experiments on the dominance or recessiveness of malignancy have mainly involved fusions between malignant cells and normal cells of different lineages. In most cases, malignancy was suppressed (Harris et al., 1969; Stanbridge et al., 1982). The availability of hybrids between cells at

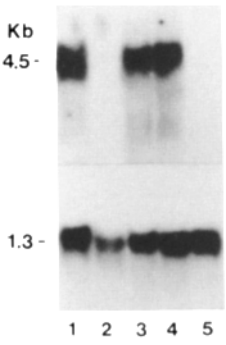

Figure 8. Northern blot showing E-cadherin mRNA levels in some parental cells and hybrids. 10 μg of total RNA was loaded in each lane. The blots were probed initially with a cDNA probe specific for E-cadherin (*top*) and subsequently with a GAPDH probe to control the level of RNA loading (*bottom*). Lane *1*, B × C5N hybrid; lane *2*, carB-5A; lane *3*, C5N; lane *4*, B × P6 hybrid; and lane *5*, B × B hybrid.

different stages of malignancy within the same lineage enabled us to ask whether the ability to suppress tumorigenicity is lost at an early stage in one step, or is progressively lost as cells proceed towards a fully malignant phenotype. The results shown in Table I suggest that the latter is in fact the case.

Hybrids between carB-5A and itself or with the other spindle cell line A5 are extremely aggressive, giving rise to spindle carcinomas with a short latency period (Fig. 9 *B* and *C* and Table I), the same as the transformed parent carB-5A (Fig. 9 *A*). Fusions with the non-tumorigenic C5N cell line resulted largely in the suppression of malignancy (Table I) although some tumors were observed after a relatively long latency period. Interestingly, these tumors were mainly high grade squamous carcinomas or spindle carcinomas and have lost E-cadherin expression (Stenback, F., unpublished results) suggesting that they may have arisen after segregation of chromosomes contributed by the C5N parental cells (Harris et al., 1969).

An intermediate pattern was seen in the hybrids with papilloma cells P6 and P1. The parental P6 and P1 cells are generally non-tumorigenic on injection sub-cutaneously into nude mice, although occasionally either benign cysts or well differentiated tumors are seen at the injection sites (Fig. 9 *D*). Strong suppression of the malignant spindle cell phenotype was seen in fusions between carB-5A cells and the P6 papilloma line. Subcutaneous lumps which developed in some mice were shown to be keratinaceous cysts lined by a layer of epithelial cells (Fig. 9, *E–G*), comparable with those generated by parental P6 cells. Similar results have been described for fusions between Hela cells and normal keratinocytes (Peehl and Stanbridge, 1981). The other papilloma cell line P1 did not cause such dramatic suppression in hybrids with carB. Although the latency for tumor development was substantially increased with respect to the carB-5A cells, some tumors did arise which were classified as squamous carcinomas at different grades of malignancy (Table I). Finally, hybrids with the squamous carcinoma line B9 consistently gave rise to tumors at the site of injection, but both the latency to tumor development (5 wk) and the tumor histology (Fig. 9 *I*) was more similar to the B9 parent (Fig. 9 *H*) rather than to carB-5A (Fig. 9 *A*). We conclude that tumorigenicity is suppressed to different levels by cells at various stages of malignancy. Some papilloma cells have the capacity to suppress the spindle cell phenotype completely, giving rise only to benign cysts, while squamous carcinomas can also induce suppression, but to a much lesser extent.

Discussion

Differentiation and Malignancy

The complex relationships between the control of cell differentiation and the establishment of the malignant phenotype have been extensively studied and reviewed (Harris, 1990; Hart and Easty, 1991). It is generally accepted that more malignant tumors differentiate poorly, and that re-establishment of the normal pattern of differentiation is as-

Table I. Tumorigencity of Hybrids and Parental Cell Lines

		Cell Morphology (in vitro)	Tumorigenicity	Latency period*	Histology‡
			positive sites/total sites injected	*wk*	
carB-5A X					
C5N	(parent§)	epi	0/16 (0%)	–	Benign cysts
	hybrids‖	epi	8/16 (50%)	9–10	Grade IV¶
P6	(parent)	epi**	1/8 (12%)	8	Benign-grade I
	hybrids	epi	4/48 (8%)	6–8	Benign-grade II
P1	(parent)	epi	3/8 (37%)	8	Benign-grade II
	hybrid	epi	4/16 (25%)	5–8	Benign-grade III
B9	(parent)	epi	8/8 (100%)	4–5	Grade I
	hybrid	epi	32/32 (100%)	4–5	Grade I–grade III
A5	(parent)	fibro‡‡	8/8 (100%)	2–3	Grade IV
	hybrid	fibro	32/32 (100%)	2–3	Grade IV
carB	(parent)	fibro	8/8 (100%)	1–2	Grade IV
	hybrid	fibro	32/32 (100%)	1–2	Grade IV

* Animals were sacrificed when tumor was greater than or equal to 1.2 cm in diameter.
‡ Grading system is based upon a grade I equivalent to a well differentiated squamous cell carcinoma up to a grade IV, which is equivalent to a spindle cell carcinoma. Several different cell morphologies were seen in an individual tumor sample. This range is indicated.
§ Parent = parental cell line used in fusions.
‖ Hybrid = hybrids generated in fusions. Values are an average from 4 independent hybrid clones.
¶ Tumors were 2–4 mm in size until 8 wk, then grew extremely rapidly until sacrifice.
** epi = epithelial morphology.
‡‡ fibro = fibroblastoid or spindle morphology.

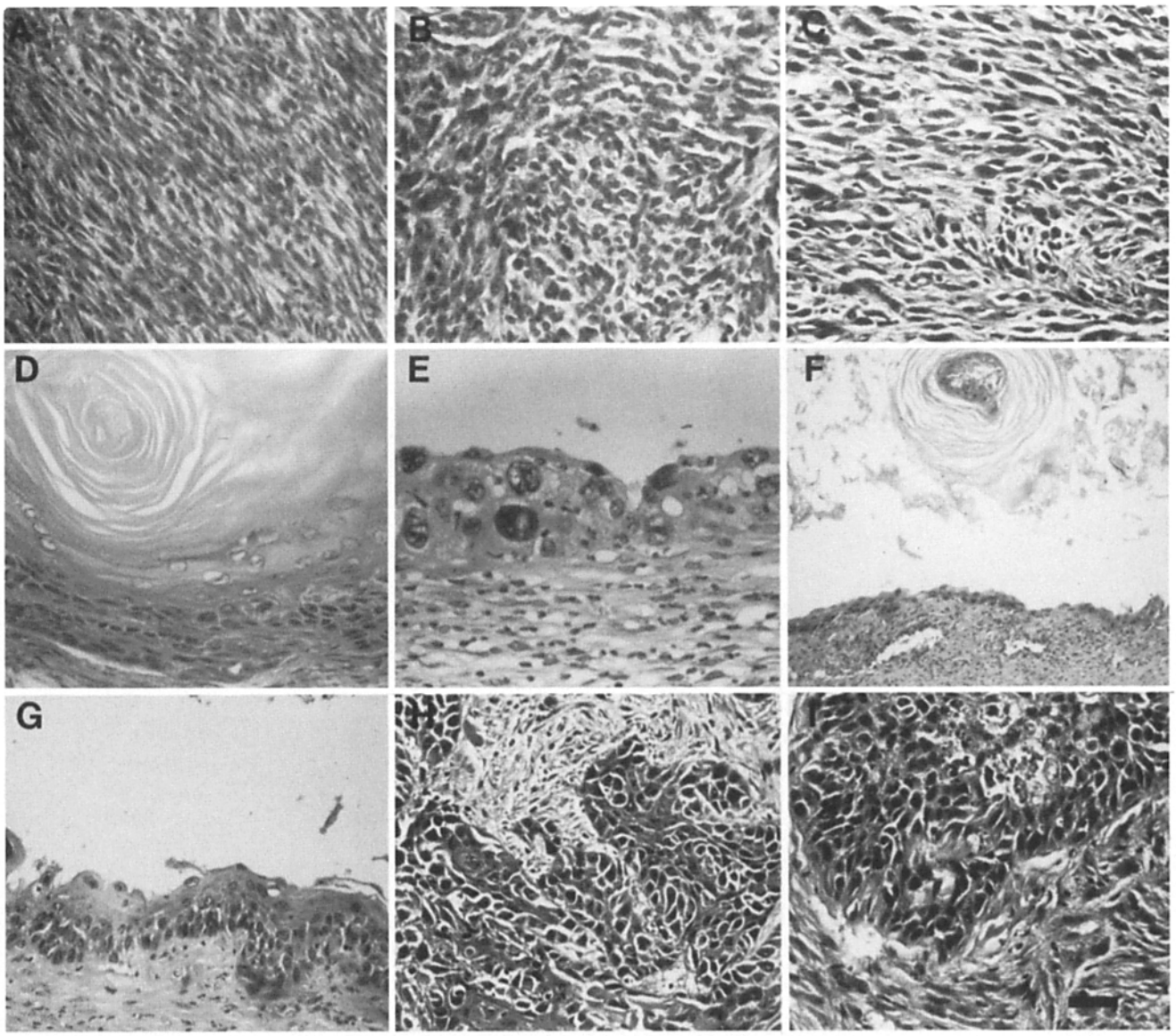

Figure 9. Histology of tumor sections. 5-μM sections of tumor were cut and stained with hemotoxylin and eosin. (*A*) Histology of tumor from carB–5A parent demonstrates a classical spindle cell carcinoma; (*B*) section from B × B hybrid tumor is a spindle cell carcinoma; (*C*) section from B × A5 hybrid shows a spindle cell carcinoma; (*D* and *E*) sections from P6 parent. The histology indicates the benign nature of the tumor, although some hyperplasia, characteristic of a benign cyst can be seen; (*F* and *G*) sections from B × P6 tumor, which again demonstrate the benign nature of the tumor; (*H*) section from B9 parent, which is characteristic of a well differentiated squamous tumor; and (*I*) section from B × B9 hybrid tumor, which has a similiar histology to the parent B9. Bars (*A–E* and *G–I*) 250 μM; (*F*) 600 μM.

sociated with tumor suppression. However, the mechanisms which influence these processes are unknown, and in particular the question of separate genetic control of differentiation and malignancy has not been fully addressed.

We have studied the relationship between differentiation and tumorigenicity by exploiting a series of cell lines derived from different stages of skin tumor progression. The parental cells have been characterized in terms of their tumorigenic properties and ability to express some of the typical epidermal differentiation markers: keratins, E-cadherin, and desmoplakin. It has previously been shown that some highly malignant epidermal cells, e.g., the spindle cell line carB, fail to express either keratins or E-cadherin (Diaz-Guerra et al., 1992; Navarro et al., 1991). Inspection of the literature shows that such observations are not uncommon. Tumors known variously as carcinosarcomas, spindle cell carcinomas or epitheliosarcomas, thought to have epithelial origins, have been described which fail to express epidermal differentiation markers and show many characteristics of fibroblastic cells (Turusov, 1979; Guldberg, 1923).

The identification of the true cell of origin of these tumors has been a controversial subject, but recently formal proof of the derivation of a spindle cell carcinoma from a preexisting squamous carcinoma was obtained by analysis of mutations present in spindle (A5) and squamous (B9) variants obtained from the same primary mouse skin tumor. These studies (Burns et al., 1991) showed that the two lines had an identical pattern of four mutations in the H-*ras* and

p53 genes, and therefore must be derived from a common precursor cell. We have shown in the present investigation that the A5 spindle line still expresses keratins and as such is probably not as far along the pathway of malignancy as the keratin-negative carB–5A cell line. Interestingly, hybrids between these two cell lines show a disorganized, punctate expression of keratin proteins, suggesting either that carB–5A cells produce a protein which positively destabilizes the keratin network, or that threshold levels of a stabilizing factor are present only in A5 cells. The concentrations of such a factor may be below threshold levels in the hybrids, with consequent disorganization of the filament network.

Neither of the two spindle cell lines used in this study expresses the E-cadherin cell adhesion molecule or the desmosomal protein desmoplakin. This suggests that these molecules may be lost before the keratin filament network is disrupted. Because of the intimate relationship between the cytoskeleton and both E-cadherin (Kemler and Ozawa, 1989), and desmosomes (Cowin et al., 1985*a*,*b*) it is possible that downregulation of adhesion protein expression may in fact contribute to the subsequent destabilization of the keratin filaments.

Is E-cadherin a Tumor Suppressor Gene?

Loss of expression of E-cadherin has been seen in a number of tumor types or cells transformed in vitro (Navarro et al., 1991; Schipper et al., 1991; Shimoyama et al., 1989; Sommers et al., 1991). This phenomenon is associated with increased invasiveness and motility of the cells (Behrens et al., 1985, 1989). Evidence that E-cadherin is directly responsible for these properties comes from experiments involving inhibition of E-cadherin function using antibodies (Behrens et al., 1985) or of its expression using antisense RNA (Vleminckx et al., 1991). The re-introduction of E-cadherin into certain cells can lead to at least partial suppression of malignancy (Navarro et al., 1991; Frixen et al., 1991).

All of this evidence suggests that E-cadherin could act as a tumor suppressor gene which is inactivated during tumor progression. The evidence from the studies described here would however suggest that while loss of E-cadherin may play a role in the late acquisition of the invasive, spindle cell phenotype, other genes are responsible for complete suppression of tumorigenicity in cell hybrids. This interpretation is based on the fact that fusion of carB–5A cells with the squamous carcinoma B9 produces hybrids which fully express E-cadherin, and which give rise to squamous carcinomas in nude mice. More complete tumor suppression is seen in fusions with cells at earlier stages of progression, independently of the level of E-cadherin expression. We conclude that E-cadherin is not the primary tumor suppressor gene which is lost in these particular cells. This interpretation is supported by recent experiments (Navarro et al., 1993) in which an E-cadherin expression vector was transfected into the carB cells used in our fusion studies. Although the exogenous gene was expressed, the protein was not stabilized at intercellular junctions and no inhibition of tumorigenicity was observed (Navarro et al., 1993). The same group has also shown that no gross alterations in the structure of the E-cadherin gene could be detected by Southern blotting using DNA from carB and other spindle cell lines (Navarro et al., 1993).

We therefore propose that the event which leads to loss of the differentiated phenotype in spindle cells corresponds to loss of a gene(s) controlling the complex epidermal differentiation program. As such, this event may be a recapitulation in tumors of the epithelial–mesenchymal transition which takes place during development (Jouanneau et al., 1991*b*).

Events Responsible for Loss of Differentiation in Squamous Carcinomas

Thiery and co-workers have shown that the squamous phenotype of certain carcinomas is unstable, and can be altered to resemble spindle cells by over-expression of growth factors such as TGF-α or acidic FGF (Jouanneau et al., 1991*a*; Gavrilovic et al., 1990). Transition to a fibroblastoid morphology is accompanied by reorganization of the cytoskeleton to express vimentin rather than the keratins (Boyer et al., 1989). Similar properties have been seen also in breast carcinoma cell lines (Sommers et al., 1989). The reversible phenotypic modifications seen in some of these carcinoma lines may occur by mechanisms similar to those which drive the progression to the spindle cell morphology in skin carcinomas. In the latter case, however, the changes appear to be irreversibly fixed, probably by genetic alterations.

A number of "candidate" genetic events could be considered responsible for the spindle cell transition. The *ras* gene itself is implicated in this process, since expression of a viral *ras* gene in MDCK cells has been shown to lead to loss of E-cadherin, and to development of more invasive properties (Darfler et al., 1986; Mareel et al., 1991). Indeed, comparison of the clonal B9 (squamous) and A5 (spindle) cell lines described here has shown that the mutant H-*ras* allele is relatively over-expressed in the A5 cells (Crombie, R., S. Haddow, C. J. Kemp, and A. Balmain, unpublished results). However, it has not been possible to reproduce the squamous–spindle conversion by over-expression of a transfected mutant H-*ras* gene in B9 cells, nor to revert spindle cells to an epithelial morphology by over-expressing the equivalent normal H-*ras* allele (Crombie, R., S. Haddow, and A. Balmain, unpublished results). These data suggest that alterations at the H-*ras* locus are not sufficient, and may not even be necessary, for conversion of squamous skin carcinomas to spindle variants.

Another candidate could be a transcription factor or other element controlling the expression of the E-cadherin and other genes required for epidermal differentiation. It has recently been reported that E-cadherin and keratin genes share at least one upstream element which constitutes a potential binding site for a common regulatory factor (Behrens et al., 1991). The series of cell lines described in this report which are derived from hybrid mice and are consequently suitable for analysis of gene loss, together with the fusion products formed between these cells and carB, are important reagents with which to study the genetic and biological basis for the loss of differentiation which accompanies the development of spindle cell tumors.

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