Polarized and Functional Epithelia Can Form after the Targeted Inactivation of Both Mouse Keratin 8 Alleles

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Abstract. We have tested the requirement of keratin intermediate filaments for the formation and function of a simple epithelium. We disrupted both alleles of the mouse keratin 8 (*mK8*) gene in embryonic stem cells, and subsequently analyzed the phenotype in developing embryoid bodies in suspension culture. After the inactivation of the mouse keratin 8 (*mK8*) gene by a targeted insertion, mK8 protein synthesis was undetectable. In the absence of mK8 its complementary partners mK18 and mK19 were unable to form filaments within differentiated cells. Surprisingly, these ES cells differentiate to both simple and cystic embryoid bodies with apparently normal epithelia. Ultrastructural analysis shows an apparently normal epithelium with microvilli on the apical membrane, tight junctions and desmosomes on the lateral membrane, and an underlying basal membrane. No significant differences in the synthesis or secretion of α_1-fetoprotein and laminin were observed between the mK8⁻ or wild-type embryoid bodies. Our data show that mK8 is not required for simple epithelium formation of extraembryonic endoderm.

NTERMEDIATE filament (IF)[1] genes constitute a superfamily whose members are expressed in very specific spatio-temporal patterns during development. Keratin IFs are found in all mammalian epithelia and are obligate heteropolymers composed of members of two groups of IF proteins, the type I and II keratins (for review see Steinert and Roop, 1988). Subsets of 2 to 10 individual keratins of the ~20 members are expressed in pairs in diverse epithelia. During mouse embryogenesis, mK8 and mK18(the mouse homologue of the human keratin 8 and 18, also named *Endo A* and *B*) are the first intermediate filament proteins to be expressed (Jackson et al., 1980; Kemler et al., 1981; Oshima et al., 1983). They are detected just before the morula stage. Subsequently, they become restricted to the simple epithelium of the early embryo, including the trophectoderm, the parietal and visceral endoderm, as well as the simple single-layered epithelia of the adult animal, such as gut and liver (Moll et al., 1982).

Keratins are commonly regarded as structural proteins. However, they differ from other cytoskeletal components by their complex and specific pattern of expression. This diversity is thought to be functionally significant. Despite several attempts to interfere with keratin filament formation, their function remains obscure. Recently, it has been reported that dominant negative mutations of keratin proteins prevented extraembryonic epithelium formation (Trevor, 1990) or resulted in a defective skin epithelium (Vassar et al., 1991).

These studies supported the generally accepted view that keratins were involved as a structural support during epithelium formation.

An important approach to studying gene function in vivo is the analysis of cells or organisms homozygous for loss-of-function mutations. Embryonic stem (ES) cells have been used to introduce targeted mutations into the germ line of mice (Thompson et al., 1989; Koller et al., 1989; Schwartzberg et al., 1989; Zijlstra et al., 1989; for review see Rossant and Joyner, 1989; Capecchi, 1989). In addition to their use for functional studies in vivo, ES cells provide a powerful in vitro model of embryonic development (Martin, 1981; Evans and Kaufmann, 1981; Doetschman et al., 1985; Nagy et al., 1990; for review, Baribault and Kemler, 1990). In suspension culture, ES cells differentiate to organized structures known as embryoid bodies (EBs) containing an outer layer of endoderm and an inner ectodermal layer, separated by a basal lamina. These EBs expand into large cystic structures reminiscent of the visceral yolk sac both morphologically and biochemically. The *mK8* type II keratin, with its partners *mK18* and *mK19* (*Endo C*) type I keratins, are induced in extraembryonic endoderm in vivo, as well as in vitro after the differentiation of ES cell and embryonal carcinoma cells to EBs (Boller and Kemler, 1983; Oshima 1981, 1982).

To analyze the function of *mK8* in early embryonic development, and to test a possible involvement of *mK8* in epithelium formation and organization, we inactivated both *mK8* alleles in ES cells, and thereby impaired filament formation for all three keratins. We analyzed the effect of the targeted mutation in developing EBs, and found that *mK8* is dispensable for the formation of extra embryonic endodermal epithelium.

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1. *Abbreviations used in this paper:* EB, embryoid body; ES, embryonic stem; HAS, hypoxanthine and azaserine; hrpt, hypoxanthine guanine phosphoribosyl transferase; h-LIF, human leukemia inhibiting factor; IF, intermediate filament; mK8, mouse keratin 8; PCR, polymerase chain reaction.

Materials and Methods

Cell Culture

E14TG2a ES cell line was originally established by Hooper et al. (1987). ES cells were routinely cultured in DME supplemented with 15% FCS, 0.1 mM β-mercaptoethanol and 1,000 U h-LIF on gelatin-coated plates. LIF was produced by transfecting the pC10-6R plasmid (kindly provided by J. Heath) (Smith et al., 1988; Moreau et al., 1988; Williams et al., 1988) into cos cells and the supernatant was subsequently titrated using ES cell-colony plating efficiency. For embryoid body formation, ES cells were plated at a density of 10^7 cells/100-mm bacterial petri dish (Fisher) in DME supplemented with 10% FBS and 0.1 mM β-mercaptoethanol. Culture medium was changed every day.

Gene Targeting

The K8TV1 targeting vector contains two arms of homology to the *mK8* gene (*Endo A al*), a 1.8-kb XbaI fragment and a 1.2 SmaI fragment, respectively. The *neo*[R] gene (pMCIneopolA, Stratagene, La Jolla, CA) replaces part of the first exon, including the ATG translation initiation codon (Sémat et al., 1988). The modified Herpes simplex virus thymidine kinase gene (Mansour et al., 1988), HSV-*tk*, was added 3', followed by the Bluescript vector (Stratagene). K8TV2 and K8TV3 are essentially identical to K8TV1 except that the *neo*[R] gene has been replaced by the pIIs(I2s) and pnII(I2s) *hprt* mini-genes (Reid et al., 1990). For electroporation, ES cells were transfected with 40 μg of Not I linearized targeting vector in 0.7 ml of culture medium using a BTX Transfector 100 at 250 V for 5 ms. Cells were plated at a density of 10^7 cells/90-mm petri dish. 12 h after electroporation, one plate was trypsinized and the number of cells counted. This number was used to calculate cell survival. 150 mg/ml G418 (for K8TV1) or G418 and hypoxanthine (0.7 μg/ml)-azaserine (10 μM) (HAS) (for K8TV2 and K8TV3) as well as 2 μM GanC were added 24 h after electroporation. One plate was selected in the absence of GanC to evaluate the transformation efficiency and the enrichment factor of the GanC selection. The number of colonies without GanC selection are deduced relative to this control. After 10 d under selection, single colonies were picked and grown in duplicate. PCR analysis was performed using 27–29-mer oligonucleotides, one complementary to the neoR gene (for K8TV1, 5' >CGC ACG GGT GTT GGG TCG TTT GTT CGG < 3'), or the HPRT gene (for K8TV2 and K8TV3, 5'-CCA CCG TGT GTT AGA AAA GTA AGA AGC AG-3') and one located 3' of the targeting vector homology in the *mK8* gene (5'-GCT GAA CAG GCA GAG AAA CTG ATT TGG GG-3'). 40 cycles of melting at 93°C for 1 min, annealing at 57°C for 30 s, and synthesis at 72°C for 2 min were performed using 100 ng/reaction of each oligonucleotide, 2 U Taq polymerase (Cetus Corp., Emeryville, CA), and crude cell lysate (~10,000 cells/reaction) (Kim and Smithies, 1988). The first screenings were done using pools of eight clones. The single clones contained in pools with the expected PCR product were rescreened with PCR for the expected 1.4-kb product. Positive clones were expanded further for Southern blot analysis. 5 μg of XbaI digested DNA per lane were loaded onto a 0.65% agarose gel and transferred to a Zeta-probe membrane (BioRad Laboratories, Cambridge, MA). The filter was hybridized with the 2.5-kb EcoRI pseudogene fragment (*Endo Aα2*) (Vasseur et al., 1985), which is homologous to the exonic sequences of the *mK8* gene.

Immunobiochemical Analysis

For immunofluorescence analysis, EBs were sedimented at 1 g, mixed with OCT compound, and frozen directly on dry ice. 5-μm sections were fixed in 100% methanol for 10 min, rinsed with PBS, and then incubated with the respective antibodies. For staining in monolayer, EBs were trypsinized, replated on gelatin-coated coverslips, and methanol-fixed 2 d later. TROMA-1, -2, and -3 rat mAbs are directed against mK8, mK18, and mK19, respectively (Boller and Kemler, 1983; Boller et al., 1987). These three hybridoma cell lines were a gift of Dr. R. Kemler (Max-Planck Institut für Immunbiologie, Freiburg, Germany). mK8 and mK18 antisera have already been described previously (Oshima, 1982). Antivimentin (Grover et al., 1983) and antidesmoplakin I/II (Pasdar and Nelson, 1991) antisera were gifts from Drs. J. Singer (University of California at San Diego, San Diego, CA) and J. Nelson (Stanford University, Stanford, CA), respectively. Laminin antisera was described previously (Oshima and Linney, 1980). Extensive immunoprecipitations and ELISA analysis confirmed that the serum was specific to the laminin A and B chains. Anti-α_1-fetoprotein was commercially available from Miles Laboratories Inc. (Elkhart, IN). Fluo-rescein-conjugated rabbit anti–rat IgG, goat anti–rabbit IgG, and rhodamine-conjugated goat anti–rabbit IgG antibodies were purchased from Organon Teknika (West Chester, PA). For double staining experiments, the species specificity of the second antibody was controlled as follows: cells were incubated with TROMA-2 rat mAb and subsequently with rhodamine anti–rabbit antisera. Also, cells were incubated with desmoplakin I/II rabbit antisera and subsequently with fluorescein-anti–rat IgG. Both controls were negative, showing that the second antibodies used here were species specific.

For most immunoprecipitation experiments, EB cultures were labeled overnight with ^{35}S-Met at a concentration of 50 μCi/ml, in methionine-free EB culture medium. Secretion was analyzed by pulse-chase experiments where cells were labeled with 200 μCi/ml of 30 min and rinsed 3× with normal complete EB culture medium. Both supernatant and EBs were collected at different chase times. Cell lysate, containing 10^7 TCA-insoluble cpm, were incubated for 2–3 h with the respective antibodies, followed by a 30-min incubation with *Staphylococcus aureus* as described previously (Oshima, 1982). The pelleted proteins were separated on a 12.5% SDS-PAGE and detected by fluorography (Bonner and Laskey, 1974). Gels were exposed to XAR-5 Kodak film. For quantitative analysis, films were scanned with an LKB laser densitometer.

Electron Microscopy

Epon-embedded EBs were sectioned and stained with uranyl acetate and lead citrate, following standard procedures for EM.

Results

Inactivation of mK8 by Gene Targeting

To impair both mK8/mK18 and mK8/mK19 keratin filament formation, we inactivated the type II mK8 keratin. In the mouse genome, there is one functional *mK8* gene with eight exons and one pseudogene (Vasseur et al., 1985). The targeting vectors used for the inactivation of both alleles are depicted in Fig. 1. They contain 3 kb of the *mK8* gene, interrupted by the *neo* gene for the targeting of the first allele (K8TV1) and by a *hprt* minigene for the targeting of the second allele (K8TV2). The Herpes simplex virus thymidine kinase, HSV-*tk*, gene was inserted to make use of the positive-negative selection with gancyclovir (GanC) (Mansour et al., 1988). E14TG2a ES cells were transfected with K8TV1 and four targeting events were identified by PCR screening (Table I). Three of those clones were expanded and the planned alteration of one *mK8* allele was confirmed by Southern blot analysis (Fig. 2).

The E14K8-N1 cell clone was used for the inactivation of the remaining allele. The parental E14TG2a ES cell line carries a deletion in the *hprt* gene and therefore lacks *hprt* activity completely (Hooper et al., 1987). Two forms of a *hprt* minigene (Reid et al., 1990) were tested as selective markers in the construction of the targeting vectors for the second allele. K8TV2 (Fig. 1) and K8TV3 (not shown) contain the *hprt* minigene including the first and second introns or the second intron alone, respectively. After transfection of E14K8-N1 cells with K8TV2 and K8TV3, *hprt*[+] clones were selected in medium containing hypoxanthine and azaserine (HAS) in order to inhibit de novo synthesis of purines and make the cells dependent upon the activity of the *hprt* gene for growth. Azaserine replaced the more conventional aminopterin and thymidine combination, found in HAT medium, to avoid possible interference with the negative selection using the nucleotide analogue GanC. G418 selection was continued during the course of the second homologous recombination experiment in order to select only recombination events with the unmodified allele. Six targeting events were identified by
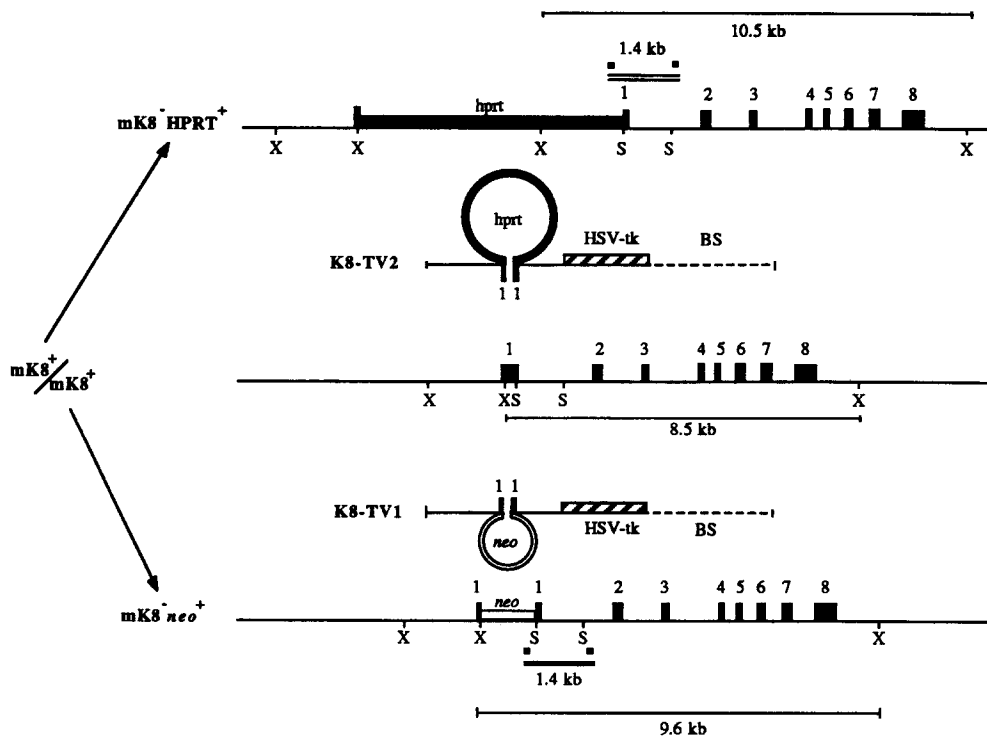
Figure 1. Targeting vectors. The *mK8* gene, represented by the middle line, contains eight exons. After homologous recombination with K8TV1 (shown below the *mK8* gene), the *neo* gene should be inserted into the first exon of the *mK8* gene, thereby deleting part of the first exon, including the ATG initiation codon, and thereby inactivates one allele, which becomes *mK8⁻* and *neo⁺*. Southern blot analysis should reveal a shift in size of the XbaI restriction fragment of one *mK8* allele from 8.5 to 9.6 kb. Small black boxes represent the oligonucleotides used to identify the targeting events by PCR (see Table I). The expected 1.4-kb PCR product is indicated by double lines. After homologous recombination with K8TV2 (shown above the *mK8* gene), the insertion of *hprt* into exon 1 of the remaining *mK8* wild-type allele should inactivate it. This allele becomes *mK8⁻* and *hprt⁺*. Southern blot analysis should reveal a shift in size of the XbaI fragment from 8.5 to 10.5 kb. X, XbaI; S, SmaI; BS, Bluescript vector.

PCR from 153 screened colonies after transfection with K8TV2, but none with K8TV3 (Table I). The targeted modifications were confirmed by Southern blot analysis for all expanded clones, which were positive by PCR (Fig. 2). One HAS[r] clone, which did not contain a second targeted gene, was also picked as a control for further functional assays (Fig. 2, lane 9).

The targeting frequency with K8TV2 (1/25 HAS[r] + GanC[r] colonies or 4.3 × 10⁻⁸ transfected cells) is approximately half of that observed with K8TV1 (1/11 HAS[r] + GanC[r] colonies or 10⁷ transfected cells). Taking into account that only half the number of targets were present after the first inactivation, these results are in agreement with previous observations that the targeting frequency is independent of the length of nonhomologous DNA (Mansour et al., 1990). The unique difference between K8TV2 and K8TV3 is the presence of the *hprt* first intron in the former construct. We were unable to detect any targeting events with K8TV3, although HAS[r] colonies were obtained. The first intron appears to contain regulatory elements necessary for sufficient levels of *hprt* expression in the context of the nonexpressed mK8 gene (Reid et al., 1990).

Table I. Targeting Frequency

	K8TV1	K8TV2	K8TV3
Transfected cells	10⁸	1.4 × 10⁸	10⁸
Surviving cells	5 × 10⁷	7 × 10⁷	5 × 10⁷
Colonies without GanC	6,400	1,526	300
Transformation efficiency	6.4 × 10⁻⁵	0.9 × 10⁻⁵	0.3 × 10⁻⁵
Colonies with GanC	320	218	30
GanC enrichment factor	20-fold	7-fold	10-fold
Screened colonies	42	153	28
PCR positives	4	6	0
Targeted clones (confirmed by Southern blot)	3/3	5/5	—
Targeting frequency per:			
GanC[r]/G418[r]/HAS[r] colonies	1/11	1/25	—
transfected cells	10⁻⁷	0.43 × 10⁻⁷	<10⁻⁸

K8TV1 targeting vector (Fig. 1) was used for the disruption of the firrst allele, K8TV2 (Fig. 1) and K8TV3 (not shown) for the disruption of the second allele. The number of surviving cells is the number of attached cells 12 h after electroporetion. The number of colonies without GanC is the number of colonies if they would have been selected in G418 (for K8TV1) or HAS (for K8TV2 and K8TV3) only. The number of colonies with GanC is the total number of G418[r] + GanC colonies (for K8TV1) or G418[r] + HAS[r] + GanC[r] colonies (for K8TV2 and K8TV3) obtained.
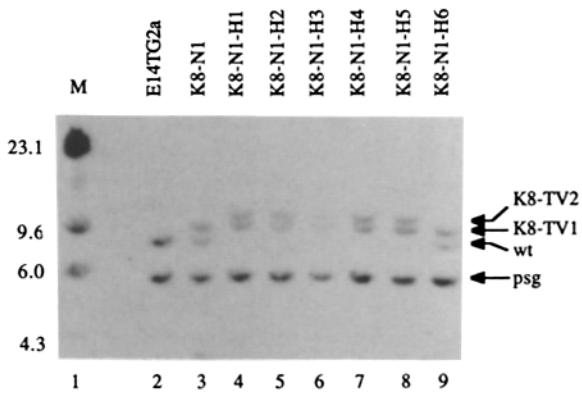
Figure 2. Southern blot analysis of the targeted ES cell clones. 5 μg of genomic DNA, digested with XbaI was loaded in each lane. The filter was hybridized with the 2.5-kb EcoRI pseudogene fragment (*Endo·Aα2* [Vasseur et al., 1985]), which is homologous to the exonic sequences of the *mK8* gene. In the parental cell line (lane 2), the 8.5 and the 5-kb bands represent the gene and the pseudogene, respectively. The presence of a 9.5-kb band in lane *3* shows the expected shift in one allele of the *mK8* gene after homologous recombination with K8TV1. The presence of the 10.5-kb band in lanes *4–8* shows that the remaining wild-type allele has been targeted with K8TV2. The control clone E14K8-N1-H6 (lane *9*) is HAS^r, but, as expected from the PCR analysis, the second allele was not targeted. Radiolabeled lambda HinDIII size markers are shown in lane 1.

## Polarized Epithelium Forms in mK8⁻ Embryoid Bodies

ES cells, homozygous or heterozygous for the targeted mutation, as well as the parental cell line were allowed to differentiate in suspension culture. All clones formed normal aggregates. Endoderm layers covering the surface of EBs were morphologically recognizable by light microscopy after 2–4 d (Fig. 3 *A*) and in histological sections (Fig. 3, *B* and *C*). Ectoderm-like epithelia formed beneath the outer endodermal layer as shown in sections of 4-d-old EBs culture (Fig. 3 *B*). Large cystic structures expanded after 8–10 d (Fig. 3 *D*). Neither the kinetics of embryoid body formation nor the proportion of aggregates that formed cystic structures appeared to be different from the wild-type in all mutant clones analyzed.

Transmission EM revealed apparently typical and normal epithelium in *mK8⁻* and wt EBs (compare Fig. 4, *A* and *B*). Cells were polarized with microvilli on the apical membrane and a basement membrane underlying the epithelial cell layer. Secretion granules were observed, indicating that these cells were actively secreting. Because keratins are associated with desmosomes we were particularly interested in whether these structures are present in *mK8⁻* EBs. Structures resembling desmosomes and tight junctions were observed on the lateral membrane of epithelial cells derived from both *mK8⁻* and wild-type ES cells (Fig. 4, *C* and *D* and *inset* to Fig. 4 *D*).

## Lack of mK8/mK18 Filaments in Doubly Targeted Clones

To confirm that the introduced mutations in the *mK8* gene functionally inactivated the *mK8* gene, we let the parental and doubly targeted ES cells differentiate to EBs and performed immunoprecipitation and immunofluorescence experiments. The mK8 was immunoprecipitated with an mK8-specific antiserum from the parental cell line (Fig. 5 *A*, lane
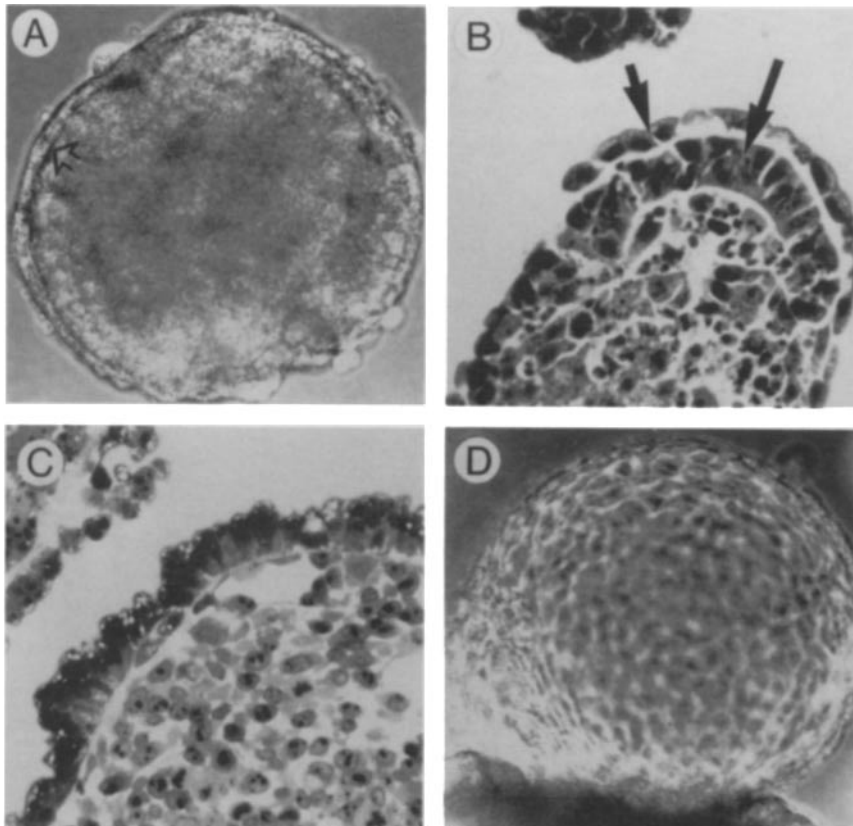
Figure 3. Embryoid body formation. Embryoid bodies (*EBs*) from the E14K8-N1-H1 doubly targeted ES cell lines are shown in *A–D*. A 4-d-old embryoid body (*EB*) is shown by phase-contrast microscopy in *A* (100×). Note the formation of an endoderm layer (*open arrow*). *B* shows a section of a paraffin embedded 4-d-old EB (250×). Note endoderm-like (*short arrow*) and ectoderm-like (*long arrow*) layers; (*C*) methylene blue–stained section of plastic embedded EB showing the differential staining of the endodermal layer (250×); *D* shows a cystic structure in a 10-d-old EB (100×).
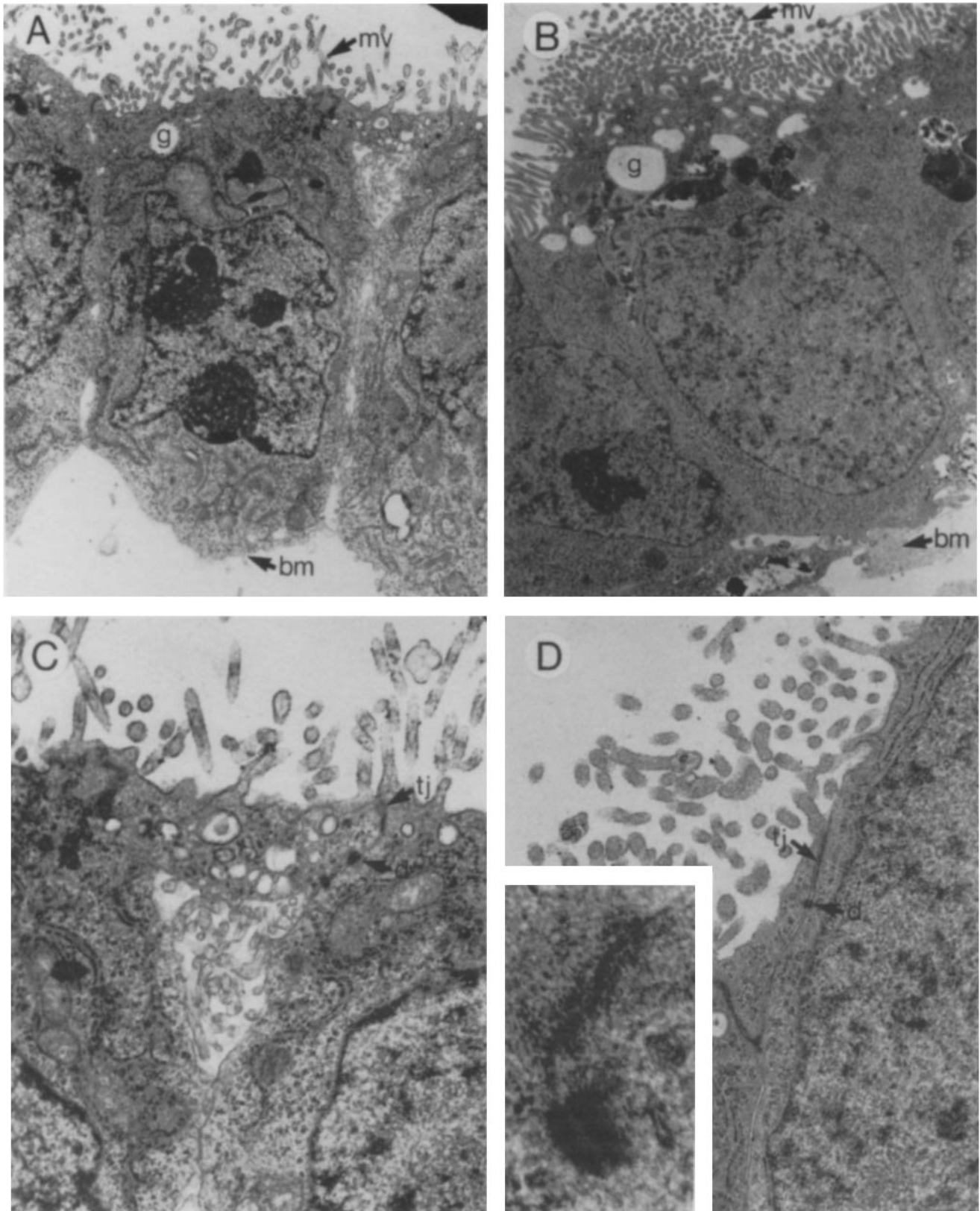
Figure 4. Ultrastructural analysis of mK8⁻ and wild-type endoderm. 4-d-old EBs derived from parental (*A* and *C*) and doubly targeted (*B* and *D*) ES cells were embedded in Epon. Transmission EM of thin sections show that mK8⁻ and wild-type EBs form a polarized epithelium with microvilli (*mv*), basal membrane (*bm*), secretion granules (*g*), tight junctions (*tj*), and desmosomes (*d*). Magnifications: (*A* and *B*) 6,000×; *C* is a higher magnification of *A* at 15,000×; (*D*) 18,000×; inset in *D* shows desomosome-like structure in mK8⁻EB at higher magnification.
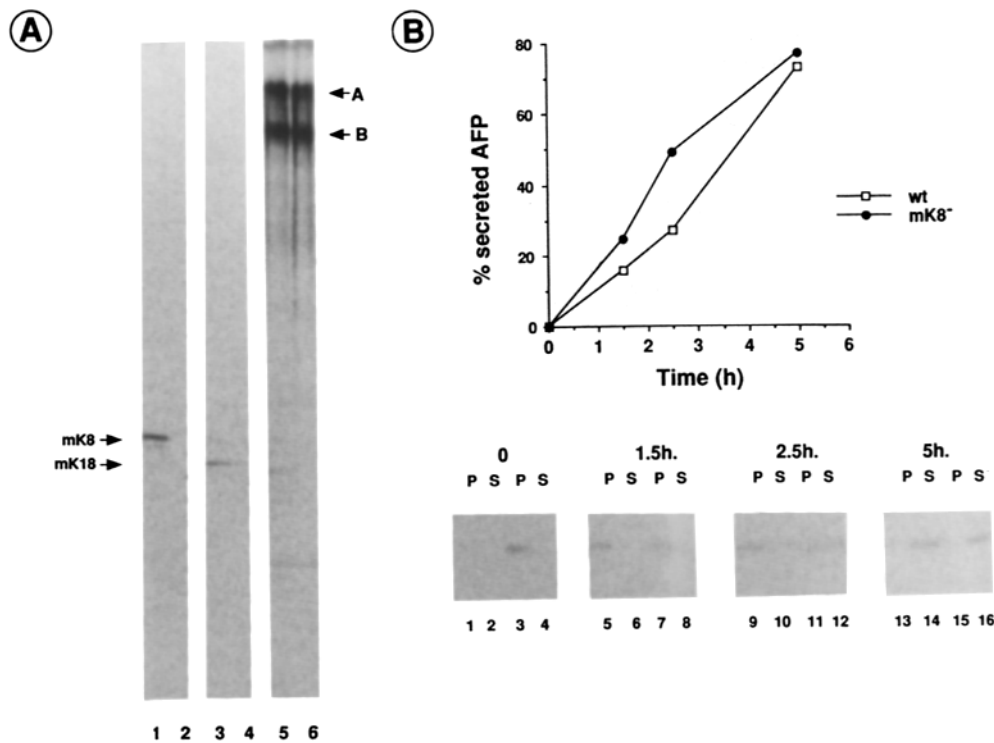
Figure 5. Immunoprecipitation of mK8, mK18, laminin, and α1-fetoprotein in wild-type and doubly targeted ES cells. (*A*) 7-d-old EBs derived from doubly targeted (lanes *2, 4, 6*) or parental (lanes *1, 3, 5*) ES cells were labeled with 50 μCi/ml ³⁵S-Met overnight. 10^7 cpm of lysate were incubated with mK8 (lanes *1* and *2*), mK18 (lanes *3* and *4*), or laminin (lanes *5* and *6*) antisera. The immunoprecipitated proteins were separated on a 12.5% SDS-PAGE and exposed to film for 3 d. Arrows indicate the position of mK8, mK18, as well as laminin A and B chains. In *B*, 12-d-old EBs derived from doubly targeted (lanes *3-4, 7-8, 11-12, 15-16*) and parental (lanes *1-2, 5-6, 9-10, 13-14*) ES cells were labeled with 200 μCi/ml for 30-min pulse. Radioactive medium was replaced with a normal medium and EBs were harvested after 0 h (lanes *1-4*), 1.5 h (lanes *5-8*), 2.5 h (lanes *9-12*), or 5 h (lanes *13-16*). α1-Fetoprotein was immunoprecipitated from the cell lysates (lanes *1, 3, 5, 7, 9, 11, 13, 15*) or from the culture supernatants (lanes *2, 4, 6, 8, 10, 12, 14, 16*). Immunoprecipitated proteins were separated on a 12.5% SDS-PAGE and exposed to film overnight. Fluorograms were scanned and the percentage of secreted α1-fetoprotein (α1-fetoprotein in supernatant/total α1-fetoprotein) was plotted as a function of time. *P*, cellular pellet; *S*, culture supernatant.

I), but was not detected in EBs derived from the doubly targeted ES cells (Fig. 5 *A*, lane 2). Immunofluorescence staining was performed on EB frozen sections. mK8 was uniformly present throughout the differentiated endoderm of wild-type EBs (Fig. 6 *A*), but was absent from doubly targeted EBs (Fig. 6 *B*). In addition, in the *mK8⁻* EBs, no mK18 staining was observed (Fig. 6, *C* and *D*), even though mK18 protein synthesis was detected by immunoprecipitation (Fig. 5 *A*, lanes *3* and *4*). These results confirm that both mK8 and mK18 are required for keratin filament formation and are in agreement with previous observations that human K18, expressed in the absence of a complementary keratin partner, is rapidly degraded (Kulesh et al., 1989). mK19 is expressed in extraembryonic endoderm and it has been suggested to be a partner keratin for mK8. mK19 was found to be present in the endoderm layer of the cystic structure in wild-type EBs (Fig. 6 *E*). In the absence of mK8, mK19 was unable to form filaments (Fig. 6 *F*), which strongly supports the notion that mK8 is the partner of mK19 in extraembryonic endoderm.

To better visualize the staining pattern of these antigens in differentiated ES cells, 10-d-old EBs were replated on coverslips. Endodermal cells as well as other cell types migrated out of the EBs. Immunofluorescence staining for mK8, mK18, and mK19 on EB outgrowths confirmed the results obtained with EB sections (Fig. 7), except for a small subpopulation of cells (from a few to about 10% of differentiated cells) which showed mK18 and mK19 staining at the periphery of *mK8⁻* cells. The nature of these cells is unclear, but

the punctate staining pattern was reminiscent of desmosome staining by desmoplakin antibodies. To test the hypothesis that the structures associated with mK18 and mK19 were desmosomes, we performed double staining of mK18 (Fig. 8 *B*) or mK19 (not shown) and desmoplakin I/II (Fig. 8 *A*). The staining patterns were identical, suggesting that in the absence of mK8, mK18 and mK19 have the ability to associate with desmosomes. The colocalization of both antigens was not observed in EB sections. Therefore, it is unlikely that this cell population represents endodermal cells. In addition, this phenomenon is not observed when ES cells are induced to differentiate to a homogenous endodermal cell population by retinoic acid (data not shown). Desmoplakin I/II is observed in most extraembryonic endoderm of both *mK8⁻* and wild-type EBs (Fig. 6, *K* and *L*).

The absence of normal filament staining patterns for mK18 and mK19 indicates that it is unlikely that another type II keratin is expressed in the differentiated cells of mK8⁻ EBs because any other type II keratin would be expected to polymerize with mK18 and mK19 resulting in detectable filamentous structures (Hatzfeld and Franke, 1985). However, vimentin is expressed in most cultured cells including undifferentiated ES cells (Paulin et al., 1982; personal observation) even though it is absent from the inner cell mass from which ES cells are derived (Jackson et al., 1980; Oshima et al., 1983). While vimentin and keratins form distinct IF networks, they are similar in primary structure (Singer et al., 1986; Steinert and Roop, 1988). Thus, vimentin could conceivably complement the absence of keratin filaments. How-

wt **mK8⁻**

mK8

mK18

mK19

ECM

Vim

DPI/II
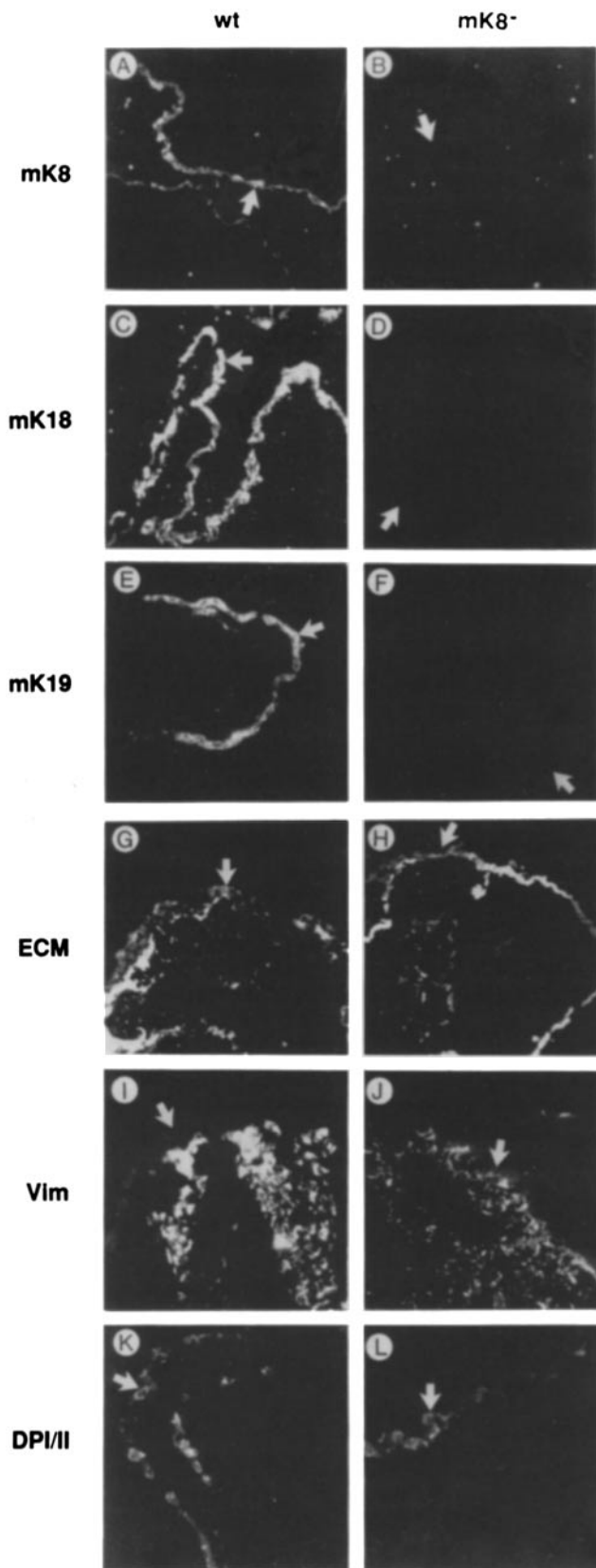
Figure 6. Immunofluorescence staining of doubly targeted and wild-type EBs. Frozen sections of EBs derived from the parental (*A, C, E, G, I, K*) and from doubly targeted (*B, D, F H, J, L*) ES cells were stained by immunofluorescence with TROMA-1 (*A* and *B*), TROMA-2 (*C* and *D*), TROMA-3 (*E* and *F*) mAbs directed against


wt **mK8⁻**

mK8
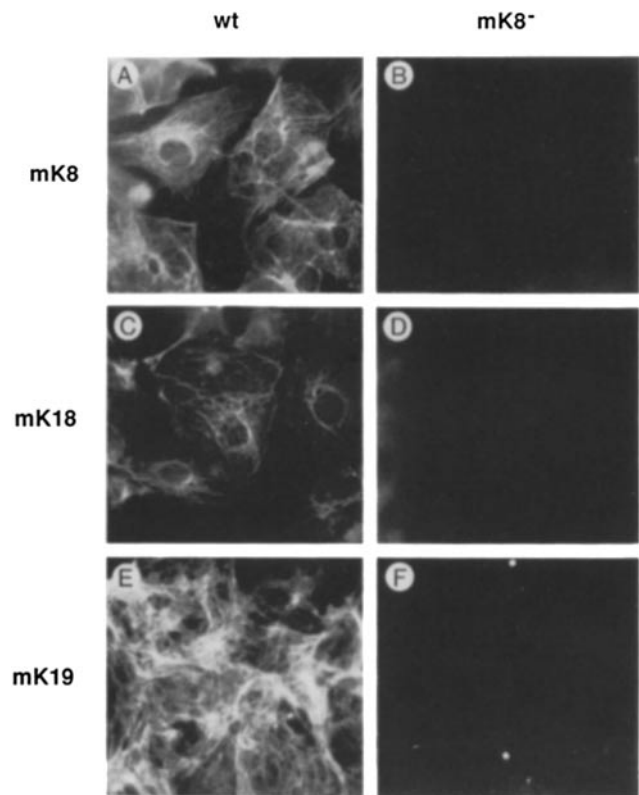
mK18

mK19

Figure 7. Immunofluorescence staining of mK8, mK18, mK19 in differentiated ES cells. 10-d-old EBs derived from parental (*A, C,* and *E*) and doubly targeted (*B, D,* and *F*) ES cells were trypsinized briefly and replated on gelatin-coated coverslips. After 3 d, cells were stained by immunofluorescence with TROMA-1 (*A* and *B*), TROMA-2 (*C* and *D*), and TROMA-3 (*E* and *F*) mAbs. (Magnification 400×.)

ever, the endodermal layer of mK8⁻ EBs was negative for vimentin staining with a polyclonal antiserum (Fig. 6, *I* and *J*).

Functional Activity of the Visceral Endoderm

The large cystic structures of EBs are reminiscent of the visceral yolk sac, both morphologically and biochemically. Visceral endoderm of the yolk sac actively produces and secretes α_1-fetoprotein and laminin in vivo. In EBs derived from embryonal carcinoma cells, the production and secretion of α_1-fetoprotein appears to be a good indicator of the organization and maturity of the visceral endodermal epithelium (Grover et al., 1983). To test the functional activity of the *mK8⁻* extraembryonic endoderm we monitored α_1-fetoprotein synthesis and secretion in EBs culture. No differences in the rate of synthesis or secretion were detectable by pulse-chase immunoprecipitation experiments (Fig. 5 *B*). Similarly, laminin synthesis (Fig. 5 *A*, lanes 5 and 6) and secretion (not shown) were not significantly different between the *mK8⁻* and wild-type EBs. Immunofluorescence

the indicated antigens, antilaminin (*G* and *H*), antivimentin (*I* and *J*), and antidesmoplakin I/II (*K* and *L*) antisera. (Magnification 250×.) The white arrows indicate endoderm layers.

DPI/II   **TROMA-2**
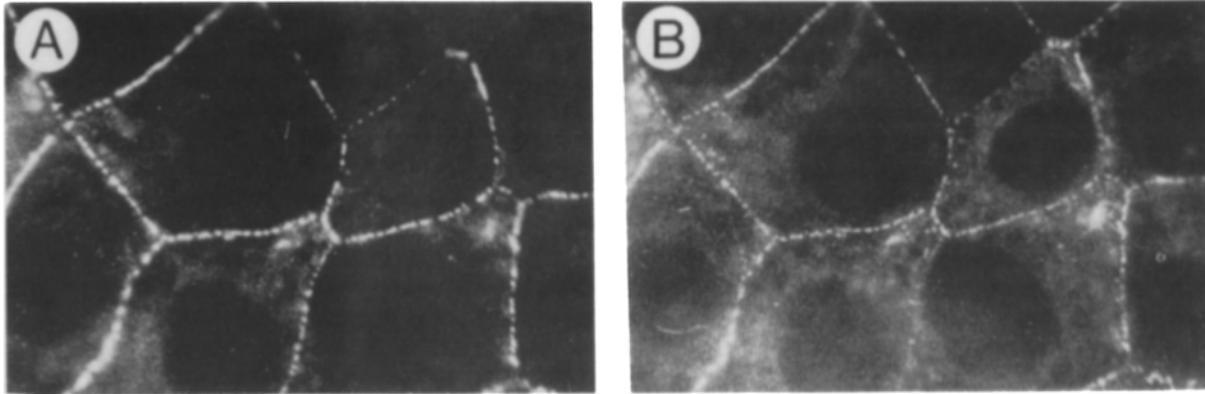
Figure 8. Double immunofluorescence staining of desmoplakin I/II and mK18 in doubly targeted differentiated ES cells. 10-d-old EBs derived from doubly targeted ES cells were trypsinized briefly and replated on gelatin-coated coverslips as in Fig. 5. Double immunofluorescence staining with antidesmoplakin I/II antisera (*A*) and TROMA-2 mAbs (*B*) show that both antigens colocalized at the periphery of the cells, in a subpopulation of epithelial cells.

staining revealed that laminin was deposited beneath the endoderm layer in mK8⁻ EBs in the same manner as in wild-type EBs (Fig. 6, *G* and *H*). These results demonstrate that an epithelium similar to the functional visceral endoderm can form in the absence of mK8.

Discussion

We show here that a polarized and functional epithelium can form in the absence of the mouse keratin 8. These results are unexpected because keratins are commonly regarded as structural proteins that provide strength to epithelial layers. However, it is clear that the visceral endodermal layer of cystic embryoid bodies that lack keratin filaments are able to withstand the apparent hydrostatic pressure associated with their expansion. In addition, the normal synthesis and secretion of α_1-fetoprotein, a sensitive marker of mature visceral endoderm, indicates that neither the processes of differentiation nor that of active secretion requires simple epithelial keratins.

Is the Lack of mK8 Requirement a Consequence of Functional Redundancy among IF Genes?

As an increasing number of targeted mutations are analyzed in mice, it has become evident that the pattern of expression of the targeted gene is not necessarily a good indicator of the tissue or time during development when the gene product is essential. The *en-2* targeted mutation has an effect restricted to neural cells expressing *en-2* but lacking *en-1* (Joyner et al., 1991). Despite constitutive expression of *c-src*, a targeted mutation of *c-src* results in a defect in bone formation only (Soriano et al., 1991). Both groups suggested that other members of the same multigene family could complement the targeted gene function when coexpressed. Functional redundancy between similar proteins has been shown to occur in the E(*spl*)-C (enhancer of *split* complex in Drosophila), where several closely related transcripts have the same expression patterns (for review see Campos-Ortega and Knust, 1990). However, it is very unlikely that the deficiency

in keratin filaments is complemented by another member of the keratin gene family, because other type II keratins capable of polymerizing with the normal mK18 and mK19 are absent from the mK8⁻ visceral endodermal layer. However, a low level or an altered polymerized form of vimentin might escape detection by immunofluorescent staining even by polyclonal antiserum and might complement the keratin deficiency. No other intermediate filament proteins are known to be expressed in this tissue.

mK8 Is Dispensable for Endoderm Formation

The results of this study appear to contrast with two recent studies of the effects of dominant-negative mutations of two different keratin proteins. The disruption of mK8/mK18 filaments by a dominant mutation of K18 impaired the formation of an extraembryonic epithelium in an EB assay similar to that used here (Trevor, 1990). However, null and dominant-negative mutations are distinct genetic modifications that can lead to distinct phenotypes. One explanation of the differences of dominant-negative keratin mutant phenotypes with the null mutant phenotype reported here is that the remaining presence of disrupted keratin filaments or bundles found in cells expressing dominant-negative mutations may interfere with components essential for epithelial assembly or integrity. Possible unexpected functions of mutant proteins and consideration of the level of expression necessary for a biological effect are complications of experiments utilizing dominant negative mutations or other similar disrupting agents. The future use of gene targeting to generate dominant negative mutations may minimize the potential complication of obtaining physiological concentrations of the mutant gene product. In a second investigation, the expression of a dominant-negative mutation of an epidermal specific keratin in transgenic mice resulted in the formation of a defective basal epithelial layer with subsequent pathology similar to a group of human genetic disorders known as epidermolysis bullosa simplex (Vassar et al., 1991). In this case, epidermal keratins appear to have an important structural function. However, epidermal and simple epithelial keratins may have

different functions. Alternatively, the differences in phenotype may be due to different requirements of the respective tissues.

The function for these simple epithelial keratins may be much more subtle than previously suggested or than revealed by the in vitro differentiation of ES cells. The analysis of germ line mutation of the keratin 8 gene will extend this analysis to all murine epithelia and provide the most rigorous test of the function of affected tissues. However, the tissue-specific patterns of keratin expression vary considerably among species. For example, the expression of keratin filaments in preimplantation mouse and hamster embryos and oocytes appears to differ significantly (Plancha et al., 1989). Myocardial and retinal pigment epithelial cells of different species differ with regard to keratin expression (Kuruc and Franke, 1988; Owaribe et al., 1988; Jahn et al., 1987; Markl and Franke, 1988). These inconsistencies suggest that keratins are fortuitously expressed in some epithelial cells. In addition, disruption of keratin filaments by antibody injection had no effect on cellular morphology or other aspects of PTK epithelial cell behavior in monolayer culture (Klymkowsky et al., 1983). Similarly, mouse embryos develop normally to the blastocyst stage after microinjection of keratin antibodies which disrupted filament organization (Emerson, 1988). These studies are in agreement with our results that mK8/mK18 and mK8/mK19 filaments are dispensable for endoderm formation.

Further Applications for Double Gene Targeting

The total loss of expression of mK8 in ES cells permits extensive biochemical analysis because EBs can be cultured in large quantity. Keratin IF bundles radiate from the perinuclear region to the desmosomes (Jones and Goldman, 1985) and hemidesmosomes (Klatte et al., 1989) which contribute to cell adhesion. Desmoplakin, a major component of the desmosome, is associated with keratin filaments before desmosome formation in MDCK cells (Pasdar et al., 1991). However, we found desmosome-like structures in the absence of mK8 filaments. Future detailed analysis of desmoplakin localization in mK8⁻ epithelial cells may reveal differences in desmosome assembly. In that respect, the colocalization of mK18 and mK19 with desmoplakin I/II in a subpopulation is a surprising observation. It seems likely that mK18 and mK19 are able to bind desmosome components without the presence of a partner keratin. A recent analysis of mK8, mK18, and mK19 domain function emphasized the necessity for the absence of the endogenous protein to perform functional and structural analysis (Xu and Lane, 1990). Using a variety of truncated keratin expression vectors ectopically expressed in fibroblasts, the authors analyzed specific domain functions in filament formation. The system developed in this study would be ideal to study the effects of these mutations in an endogenous epithelial cell where keratins could interact with desmosome components.

The method of targeting both alleles of a gene and analyzing the phenotype in vitro (Riele et al., 1991; Mortensen et al., 1991) may be suitable for any gene that is not essential for ES cell growth and is expressed in differentiated ES cells such as the early embryonic development lineages, hematopoietic lineages (Schmitt et al., 1991; Wiles and Keller, 1991), and cardiac muscle cells (Robbins et al., 1990). In addition, in cases where targeted mutations are embryonic le-

thal, chimeras of doubly targeted ES cells with wild-type embryos will permit the analysis of the phenotype at later stages (Pevny et al., 1991).

hprt is a bidirectional selective marker (Reid et al., 1990; Valancius and Smithies, 1991). We selected for the presence of HPRT activity. Subsequent selection against HPRT with thioguanine would permit replacement of the modified gene with additional constructs carrying modification of protein domains or regulatory signals. This will permit unlimited modifications of one allele in a null genetic background.

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