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Members of the *src* and *ras* Oncogene Families Supplant the Epidermal Growth Factor Requirement of BALB/MK-2 Keratinocytes and Induce Distinct Alterations in Their Terminal Differentiation Program

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BALB-/MK-2 mouse epidermal keratinocytes required epidermal growth factor for proliferation and terminally differentiated in response to high Ca^{2+} concentration. Infection with retroviruses containing transforming genes of the *src* and *ras* oncogene families led to rapid loss of epidermal growth factor dependence, in some cases, accompanied by alterations in cellular morphology. The virus-altered cells continued to proliferate in the presence of high levels of extracellular calcium but exhibited alterations in normal keratinocyte terminal differentiation that appear to be specific to the particular oncogene. These alterations bore similarities to abnormalities in differentiation observed in naturally occurring squamous epithelial malignancies.

An approach toward understanding the genetic alterations that cause normal cells to become neoplastic has come from studies of acute transforming viruses. These viruses have substituted discrete segments of host genetic information for viral genes necessary for replication. When incorporated within the retroviral genome, such transduced cellular (*onc*) sequences acquire the ability to induce neoplastic transformation (6). Of approximately two dozen isolates, several have incorporated the same *onc* sequence. Moreover, there is accumulating evidence that genetic and functional relationships also exist among a number of *onc* genes derived from different cellular loci (14). One major *onc* gene family consists of at least five distinct tyrosine kinase coding members, whereas another, the *ras* gene family, contains three highly conserved genes (14).

We have reported the development of clonal mouse epidermal keratinocyte (BALB/MK-2) cells, which require epidermal growth factor (EGF) for their proliferation and terminally differentiate in response to an extracellular calcium (Ca^{+}) concentration greater than 1.0 mM (34). These and other approaches have made it possible to study the effects of acute transforming viruses on culture epithelial cells, the most common targets for neoplastic alterations in vivo (12, 34, 37). In the present studies, we explored the effects of *onc* genes of the tyrosine kinase family on the growth and differentiation of BALB/MK-2 cells and compared the alterations they induced with those induced by *ras* transforming genes (34). We demonstrate that two major families of oncogenes supplant the EGF requirement for keratinocyte growth. We further establish that the interactions of different *onc* genes with these cells lead to blocks at distinguishable steps in the keratinocyte differentiation pathway.

MATERIALS AND METHODS

Cell culture. BALB/3T3 embryo fibroblasts (2) were maintained in Dulbecco modified Eagle medium (MEM) (Associated Biomedic Systems, Inc.) supplemented with 10% calf serum (Colorado calf serum). BALB/MK-2 cells were grown in MEM containing a calcium concentration of 0.05 mM and supplemented with 10% dialyzed fetal calf serum (GIBCO Laboratories) and 4 ng of EGF (Collaborative Research, Inc.) per ml. All cells were negative for the presence of mycoplasma by culture techniques.

Virus and virus transformation assays. Mouse type-C helper leukemia viruses included clonal strains of Rauscher murine leukemia virus (MuLV) and Moloney MuLV (24). MuLV pseudotypes of Abelson MuLV, Kirsten murine sarcoma virus (MSV), Moloney MSV, M Rous sarcoma virus (RSV), and ST feline sarcoma virus (FeSV) were obtained by superinfection of appropriate nonproducer cell lines (1). M RSV was a gift of S. Anderson (Merck Sharp & Dohme).

Titers of transforming viruses were assayed by focus formation on BALB/3T3 cells as previously described (1). For virus infection of keratinocytes, cells were seeded overnight at a density of 5×10^5 cells per dish in growth medium plus 2 μg of Polybrene per ml. After exposure to serial virus dilutions, the cells were incubated overnight and refed with growth medium minus EGF. Foci of virus-transformed keratinocytes appeared between 1 to 2 weeks later. Representative foci were removed from dishes and recloned by the limiting dilution technique for further characterization.

Radioimmunoprecipitation of keratin. Growing cultures in 100-mm dishes were incubated overnight in methionine-free MEM supplemented with 10% fetal calf serum with 80 μCi of [^{35}S]methionine per ml. The cells were rinsed with 1 mM Tris (pH 7.6), removed with a rubber spatula, and lysed by homogenization. After centrigufation, the pellet was rinsed twice with 2.0% Nonidet P-40 (NP-40) and then dissolved in 2.0% sodium dodecyl sulfate (SDS) with 10 mM dithiothreitol. The samples were incubated at 37°C for 15 min, boiled for an additional 2 min, and centrifuged. The supernatant was analyzed for the presence of keratin by the radioimmunoprecipitation procedure of Kessler (15), with antiserum against murine keratins provided by S. Yuspa. SDS-

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polyacrylamide gel electrophoresis was performed by the method of Laemmli (18).

Radioimmunoprecipitation of bullous pemphigoid. Cells were seeded in 100-mm petri dishes at a concentration of 10^6 cells per dish in low-calcium (0.05 mM) growth medium. When the dishes were ~90% confluent, calcium chloride was added to half the dishes to give a final concentration of 1.0 mM. Three days later, cultures were rinsed once with phosphate-buffered saline (PBS) and incubated overnight in methionine-free MEM with the appropriate concentration of calcium plus 10% dialyzed fetal calf serum and 70 μCi of [^{35}S]methionine per ml. The cells were then dissolved in a Tris buffer (pH 7.4) containing 0.5% NP-40. After centrifugation, the supernatants were dialyzed against three changes of Tris buffer (pH 7.4) containing 0.3% NP-40. Samples were then preincubated with normal human serum for 2 h at 0°C with equal counts per minute per sample. Antibody-bound proteins were removed by incubation of freshly prepared Pansorbin (Calbiochem-Behring), and the samples were incubated overnight at 0°C with the appropriate antiserum. Human serum reactive with pemphigoid antigen (Ag) was generously provided by John Stanley. Antibody-bound proteins were again precipitated with freshly prepared Pansorbin, and samples were analyzed by SDS-polyacrylamide gel electrophoresis.

Immune immunofluorescence of pemphigus Ag. The presence of the pemphigus Ag in both uninfected and infected cells was visualized by indirect immunofluorescence techniques (29). Cells were seeded onto cover slips at a density of 3×10^4 cells per cover slip in low-calcium (0.05 mM) growth medium and allowed to reach ~90% confluence. Some cultures were then supplemented with calcium chloride to give a final concentration of 1.0 mM. Three days later, the cover slips were stained directly with human antiserum against pemphigus vulgaris for 30 min at 37°C. The cover slips were then rinsed three times with PBS and treated with fluorescein-conjugated goat anti-human immunoglobulin G (National Institutes of Health) and incubated for 30 min at 37°C. After three rinses with PBS and one rinse with double-distilled water, the cover slips were inverted and mounted on glass slides with a solution of glycerol-PBS (1:1). Samples were viewed on a Zeiss photomicroscope fitted with a Ploem UV system.

Determination of Ulex Europaeus I binding. The ability to bind the lectin Ulex Europaeus I by both uninfected and infected cells was determined by direct immunofluorescent techniques (23). Cells were seeded onto cover slips at a density of 3×10^4 cells per cover slip in low-calcium (0.05 mM) growth medium and allowed to reach ~90% confluence. Some cultures were then supplemented with calcium chloride to give a final concentration of 1.0 mM. Three days later, cover slips were stained directly with fluorescein-conjugated Ulex Europaeus I lectin (Vector Laboratories) for 30 min at room temperature. After three rinses with PBS and one rinse with double-distilled water, the cover slips were inverted and mounted on glass slides with a solution of glycerol-PBS (1:1). Samples were viewed on a Zeiss photomicroscope fitted with a Ploem UV system.

RESULTS

Growth alterations in BALB/MK-2 cells induced by transforming genes of the *src* family. The oncogenes of RSV (*src*), Abelson MuLV (*abl*), and ST FeSV (*fes*) are related at the nucleotide sequence level, and each encodes a protein with tyrosine kinase activity (14). The oncogene product of Moloney MSV *mos* lacks detectable tyrosine kinase activity, but *mos* shares sequence homology with these genes (20, 33). We initially sought to determine whether *onc* genes of the *src* family could alter the EGF requirement for BALB/MK-2 proliferation. Each of these viruses induced the growth of EGF-independent foci in infected BALB/MK-2 cultures (Table 1). The efficiency of EGF-independent focus formation was 10- to 100-fold lower than that observed for induction of transformed foci on BALB/3T3 cultures. The lack of any detectable effect of the helper virus alone, as well as the one-hit titration patterns observed, implied that the growth alterations induced in BALB/MK-2 cells were the direct result of infection with each of these acute transforming viruses. As previously reported, *ras*-oncogene-containing viruses, Harvey and Kirsten MSV, also induced EGF-independent foci (34; Table 1). Thus, representatives of two major oncogene families supplanted this growth factor requirement of epidermal keratinocytes.

Figure 1 shows the appearance of EGF-independent colonies induced by each of the viruses analyzed. Kirsten (Fig. 1b) and Moloney (Fig. 1c) MSV-induced foci were most difficult to distinguish from foci of EGF-stimulated, uninfected BALB/MK-2 cells (Fig. 1a). M RSV, ST FeSV, and Abelson MuLV induced more readily detectable cellular alterations. Cells within M RSV and ST FeSV foci tended to grow more densely, and many were altered and elongated (Fig. 1d and e). Abelson MuLV infection was associated with the most striking changes. There was a loss of epithelial cell morphology, resulting in the appearance of foci comprised entirely of densely growing, rounded cells (Fig. 1f). These findings suggested that different acute viruses might be altering the properties of the epidermal keratinocytes in biochemically distinguishable ways.

Calcium-induced terminal differentiation of BALB/MK-2

TABLE 1. Focus formation by acute transforming retroviruses on BALB/MK-2 and BALB/3T3 cells

Virus[a]	Oncogene	Virus titer on the following cell lines (focus-forming units/ml):	
		BALB/MK-2[b]	BALB/3T3[c]
M MuLV		<10	<10
R MuLV		<10	<10
Abelson MuLV (M MuLV)	v-*abl*	1.0×10^2	8.0×10^3
M RSV (M MuLV)	v-*src*	1.0×10^4	3.0×10^6
Snyder-Theilen FeSV (R MuLV)	v-*fes*	1.6×10^1	2.0×10^3
Moloney MSV (M MuLV)	v-*mos*	5.0×10^2	4.0×10^4
Harvey MSV (R MuLV)	v-H-*ras*	1.0×10^5	2.0×10^6
Kirsten MSV (M MuLV)	v-K-*ras*	3.0×10^4	2.0×10^6

[a] Mouse type C helper leukemia viruses included clonal strains of Rauscher MuLV (R MuLV) and Moloney MuLV (M MuLV) (24, 30). Moloney and Rauscher MuLV pseudotypes of each transforming virus, indicated by parenthetical M MuLV and R MuLV, respectively, were obtained by superinfection of appropriate nonproducer cell lines (1).

[b] BALB/MK-2 epidermal keratinocytes were maintained in MEM (GIBCO) with a reduced level of calcium (0.05 mM) supplemented with 8% chelex-treated fetal calf serum (Reheis) plus 4 ng of EGF per ml (Collaborative Research) (34). Titers of transforming viruses were obtained for induction of EGF-independent foci on BALB/MK-2 cells as previously reported (34) with the following modifications. The cells were seeded overnight at a density of 5×10^5 cells per dish in medium containing 0.05 mM calcium plus 4 ng of EGF per ml. After exposure to virus, EGF was removed from the medium 24 h later. Foci were scored at 10 days after infection. At this time, uninfected BALB/MK-2 cells remained as a viable, nonproliferative monolayer.

[c] BALB/3T3 embryo fibroblasts were maintained in Dulbecco modified MEM (GIBCO) supplemented with 10% calf serum (Colorado calf serum) (1). Sarcoma virus titers on BALB/3T3 cells were assayed as previously described (24).

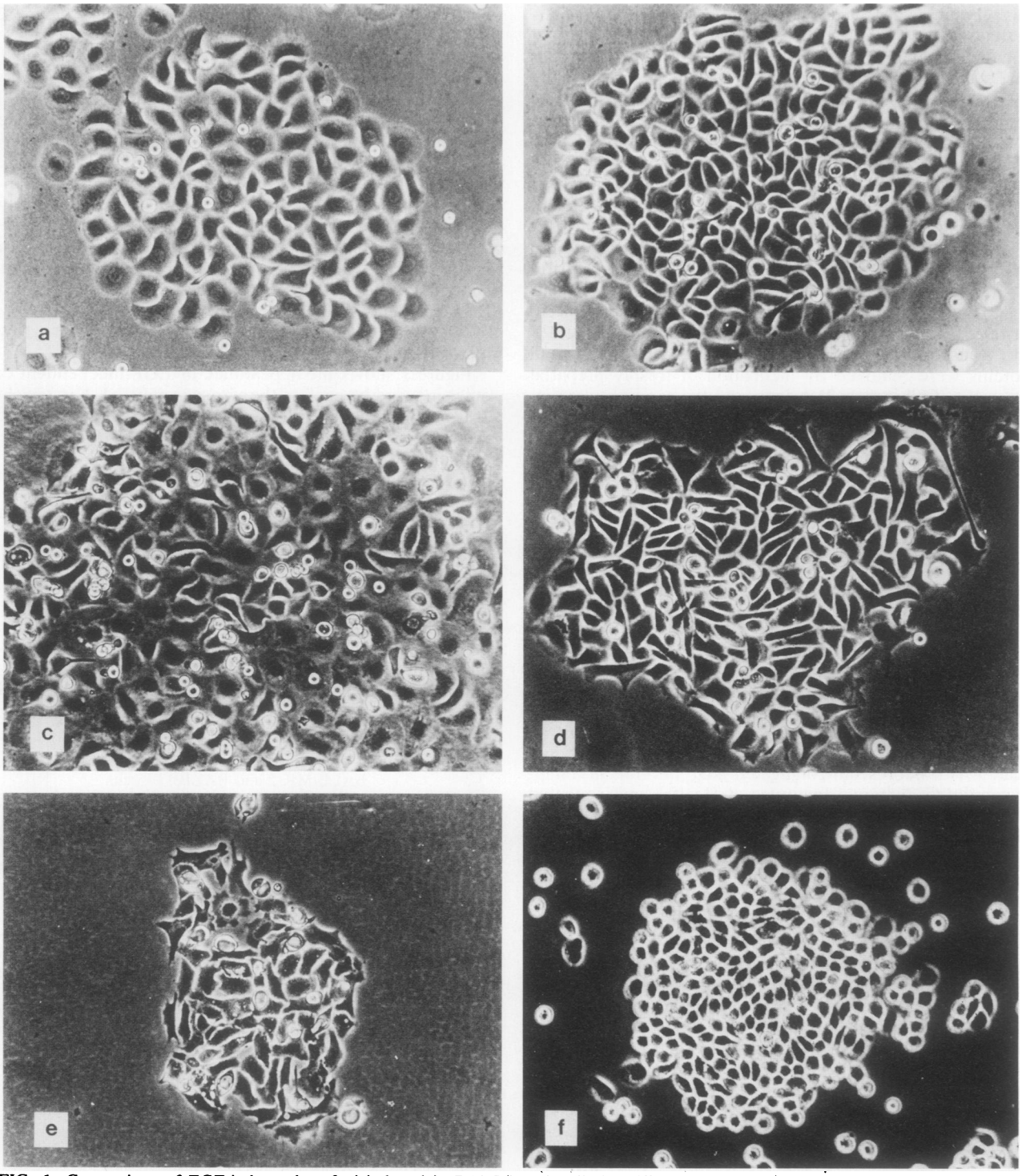


FIG. 1. Comparison of EGF-independent foci induced in BALB/MK-2 cells by different acute transforming retroviruses. After virus infection, the cells were maintained in low-calcium growth medium in the absence of EGF. Typical foci induced by each virus are shown in uninfected cells and in infected cells at 2 weeks after infection with Kirsten MSV (b), Moloney MSV, (c) ST FeSV, (d) M RSV, (e) and Abelson MuLV (f).

cells is blocked by *onc* genes of the tyrosine kinase family. To characterize their growth properties, clonal virus-altered cell lines induced by each virus were isolated. Each cell line was tested and shown to release the appropriate parental acute virus. Failure to undergo calcium-induced germinal differentiation is a property that has been shown to be associated with malignant transformation of murine epidermal keratinocytes (17, 36) and with infection by *ras*-containing retroviruses (34, 37). In the presence of high levels of Ca^{2+}, uninfected BALB/MK-2 cells ceased to divide and under-

TABLE 2. Growth properties of acute retrovirus-infected BALB/MK-2 cell lines

Cell line	Colony-forming efficiency with[b]:			
	Low Ca^{2+}		High Ca^{2+}	
	+EGF	−EGF	+EGF	−EGF
Uninfected	3.0	<0.01	<0.01	<0.01
Abelson MuLV (M MuLV) infected				
Clone 1	3.0	2.5	4.0	2.0
Clone 2	2.5	2.5	3.0	2.5
Clone 3	3.5	3.0	2.5	3.0
M RSV (M MuLV) infected				
Clone 1	0.1	0.1	0.5	1.0
Clone 2	0.3	0.1	0.1	0.2
ST FeSV (R MuLV) infected				
Clone 1	10.0	10.0	9.5	9.0
Clone 2	6.0	7.0	7.0	7.5
Clone 3	6.0	6.0	6.0	7.0
Moloney MSV (M MuLV) infected				
Clone 1	1.0	0.5	1.0	1.0
Clone 2	3.5	3.0	4.5	4.0
Clone 3	4.0	3.0	4.0	4.5
Kirsten MSV (R MuLV)				
Clone 1	4.0	4.0	6.0	6.0
Clone 2	5.0	4.0	7.5	7.5
Clone 3	4.0	2.0	6.5	6.5

[a] M MuLV, Moloney MuLV; R MuLV, Rauscher MuLV.

[b] Colony-forming efficiency was measured by plating cells in low-calcium growth medium at serial 10-fold dilutions. After overnight incubation, the cells were refed with low-calcium growth medium. In some cases, the medium was supplemented with 4 ng of EGF per ml 1.5 mM $CaCl_2$ or both. Two weeks later, the dishes were ethanol fixed and Giemsa stained. Colonies containing more than 100 cells were scored as positive. The results represent the number of colonies per number of cells plated × 100% and reflect the mean results of two independent experiments. +EGF, Supplemented with EGF; −EGF, nonsupplemented.

went terminal differentiation (Table 2). When each of the virus-altered BALB/MK-2 cell lines was exposed to medium containing 1.0 mM calcium, it continued to proliferate with cloning efficiencies indistinguishable from those of the cells propagated at low levels of Ca^{2+} (Table 2). M RSV-altered cells exhibited somewhat lower colony-forming efficiencies under both permissive and nonpermissive conditions. Thus the *src* gene appeared to be associated with an as yet unexplained generalized decrease in keratinocyte viability.

Figure 2 compares the appearance of the virus-altered cells in the presence of high calcium. Kirsten and Moloney MSV-infected BALB/MK-2 cells (Fig. 2b and c) maintained a relatively differentiated appearance, showing stratification and loss of distinct cell borders similar to that of the BALB/MK-2 parent (Fig. 2a). M RSV- and ST FeSV-altered keratinocytes responded to high levels of calcium by flattening but showed little evidence of stratification (Fig. 2d and e). Finally, there was no obvious effect of high levels of calcium on the appearance of Abelson MuLV-infected BALB/MK-2 cells (Fig. 2f). These results indicated that acute transforming viruses blocked keratinocyte terminal differentiation but supported the concept that the alterations induced were distinguishable.

Expression of epidermal differentiation markers in retrovirus-altered BALB/MK-2 cell lines. A number of markers associated with the pathways of keratinocyte differentiation have been characterized. Keratins comprise the intermediate filaments in all epithelial cells. Some keratin species appear to be expressed in all types of epithelial cells, while a 67,000-dalton species is expressed only in stratified epithelium in vivo but not in vitro (27). Pemphigoid Ag and pemphigus Ag have been isolated from the sera of human patients who have developed autoimmune diseases of the skin (5). One group of such sera recognizes an antigen present in the basal cell layer (pemphigoid Ag), while a second binds the spinosum and the granulosum layers of the epidermis (pemphigus Ag). Previous studies have shown that pemphigoid Ag is present only in primary mouse epidermal keratinocytes cultured in low levels of extracellular calcium, while pemphigus Ag is expressed only when the cells are switched to high calcium concentration (29). Another biochemical marker for differentiation in epidermal cells is preferential lectin binding. Different layers of the epidermis are capable of binding only certain lectins. It has been shown that Ulex Europaeus I lectin binds specifically to cells of the stratum granulosum (23). Therefore, as with the pemphigus Ag marker, Ulex Europaeus I binding is observed with normal epidermal keratinocytes only in the presence of high extracellular levels of calcium.

Many tumors exhibit abnormal expression of differentiated functions, and infection of some cell types with acute transforming retroviruses has been shown to lead to less differentiated phenotypes (4, 34). Thus, we examined BALB/MK-2 cell lines altered by different retroviral *onc* genes for their expression of differentiation markers under conditions of growth at low calcium concentration. There was no detectable loss or alteration of the expression of keratin species in any of the cell lines examined (Fig. 3). Moreover, like the uninfected BALB/MK-2 cells, each virus-altered cell line expressed pemphigoid Ag when cultured at low levels of Ca^{2+} (Fig. 4). While expression of pemphigus Ag was not observed in BALB/MK-2 cells (Fig. 5a) or with most of the virus-altered cell lines, represented by Abelson MuLV- and ST FeSV-infected cells (Fig. 5 c and e, respectively), the Kirsten MSV- (Fig. 5g) and the Moloney MSV-infected BALB/MK-2 cells (not shown) displayed the antigen in a small percentage of cells. At low levels of Ca^{2+}, neither uninfected cell lines nor any of the virus-altered cell lines demonstrated any binding by the Ulex Europaeus I lectin as shown in representative cell lines (Fig. 6a, c, and e).

High Ca^{2+} challenge to retrovirus-altered BALB/MK-2 cells unmasks distinct alterations in expression of epidermal differentiation markers. The time course of BALB/MK-2 cell terminal differentiation in response to high Ca^{2+} challenge is between 7 to 10 days. Cornified envelopes, a marker of terminally differentiated keratinocytes (31), can be detected as early as 3 days (34). To ascertain whether the failure of retrovirus-altered BALB-MK-2 cells to terminally differentiate was associated with altered expression of epidermal differentiation markers, we examined virus-infected lines at times up to 7 days after exposure to high levels of Ca^{2+}. Whereas at least 20% of BALB/MK-2 cells had terminally differentiated by this point, less than 0.1% of cells within any of the virus-altered lines demonstrated cornified envelope formation. These results were consistent with our findings that the virus-altered lines showed no reduction in their colony-forming efficiency in high levels of Ca^{2+} (Table 2).

To examine biochemical and immunologic markers of epidermal cell differentiation, we chose to analyze cells three days after Ca^{2+} challenge, a time at which these markers had become stably altered in BALB/MK-2 cells. The results with each of the virus-altered cells analyzed at later times after exposure to high levels of Ca^{2+} were similar. Ca^{2+} challenge led to a significant decrease in pemphigoid Ag expression by the parental BALB/MK-2 cell line (Fig. 4, lanes a and b). The cells also showed a striking increase in pemphigus Ag

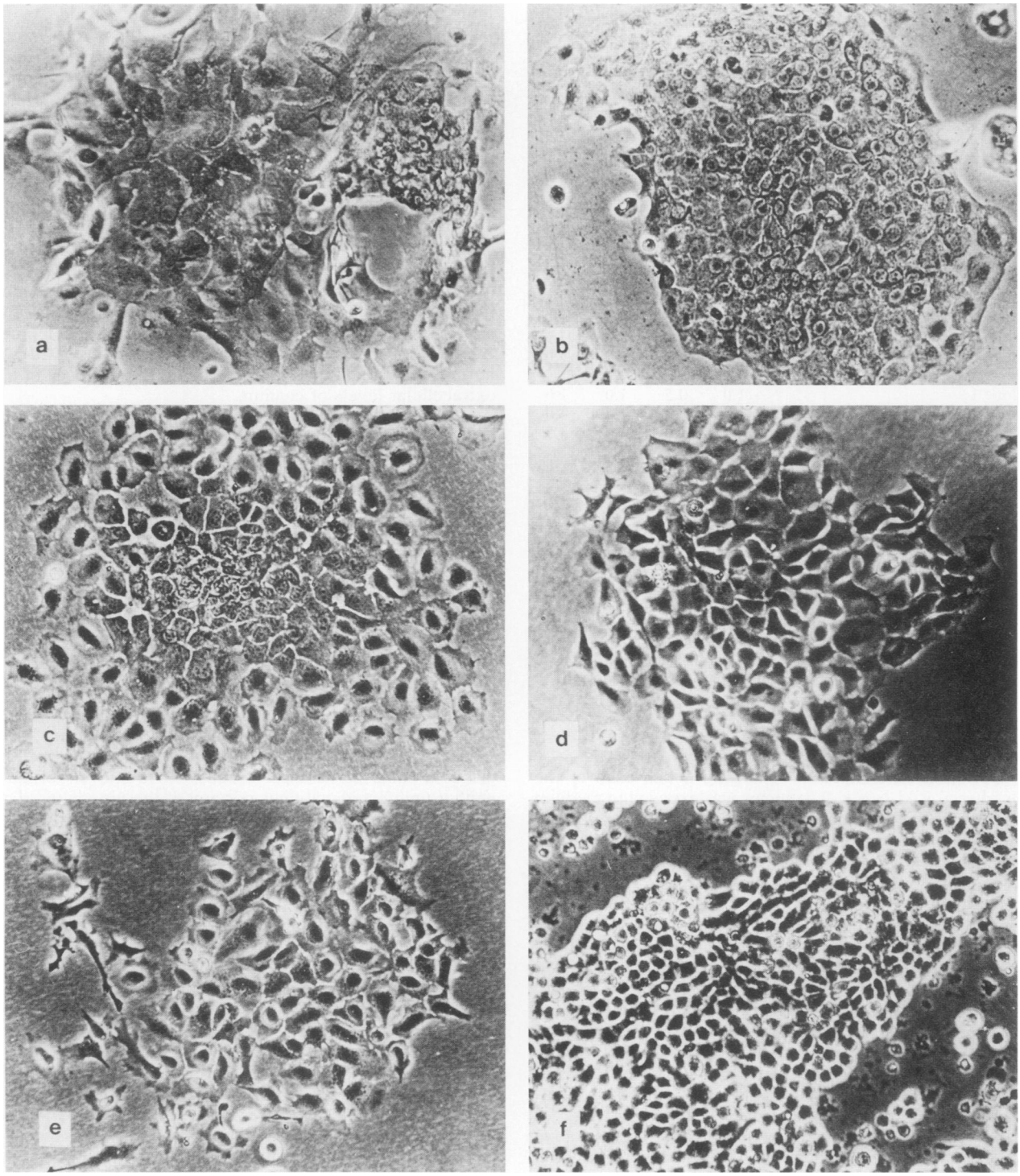


FIG. 2. Comparison of acute-retrovirus-infected BALB/MK-2 cells after exposure to high-calcium growth medium (1.5 mM Ca^{2+}). Uninfected or virus-altered BALB/MK-2 cells were seeded at a density of 10^6 cells per 100-mm petri dish in low-calcium growth medium. Four days later, the cells were switched to high-calcium growth medium. Typical colony morphology for each cell line after 72 h of exposure to high-calcium growth medium is shown in uninfected cells (a), and in cells infected with Kirsten MSV (b), Moloney MSV (c), ST FeSV (d), M RSV (e), and Abelson MuLV (f).

expression (Fig. 5b) and the ability to bind the lectin (Fig. 6b). Thus BALB/MK-2 cells appeared to differentiate in a manner analogous to that of normal epidermal keratinocytes (29).

We observed differences in the expression of these same markers among the virus-altered BALB/MK-2 cell lines exposed to high levels of Ca^{2+}. Abelson MuLV-infected BALB/MK-2 cells exhibited the least differentiated pheno-

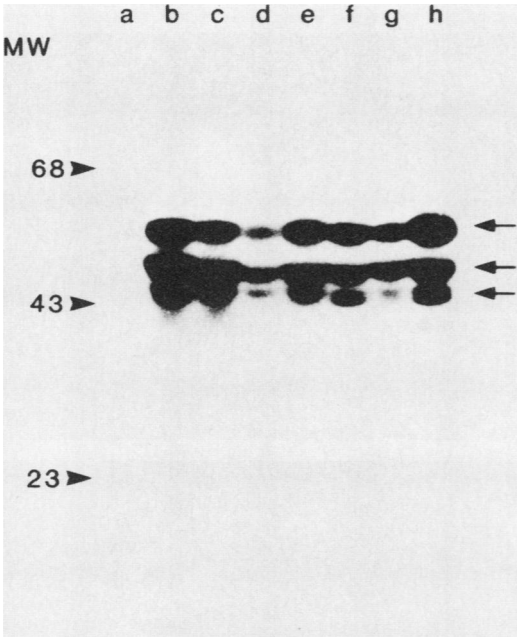


FIG. 3. Keratin synthesis by uninfected or virus-altered BALB/MK-2 cells. Growing cultures in 100-mm petri dishes were incubated for 3 h in methionine-free MEM with dialyzed fetal calf serum plus 80 μCi of [^{35}S]methionine per ml. The cells were rinsed with 1 mM Tris (pH 7.6) and lysed with 2.0% NP-40. After centrifugation, the pellet was rinsed twice with 2.0% NP-40 and then dissolved in 2.0% SDS plus 10 mM dithiothreitol. The samples were incubated for 15 min at 37°C and pelleted by centrifugation. Supernatants were analyzed for the presence of keratin species by the radioimmunoprecipitation method of Kessler with antiserum against murine keratins (15). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18). Cell extracts from [^{35}S]methionine-labeled BALB/3T3 (a), BALB/MK-2 (b), Pam 2-12 (c), Abelson MuLV-infected BALB/MK-2 clone 1 (d), Abelson MuLV-infected BALB/MK-2 clone 2 (e), ST FeSV-infected BALB/MK-2 (f), Moloney MSV-infected BALB/MK-2 (g), and Kirsten MSV-infected BALB/MK-2 (h) cells were immunoprecipitated with antikeratin serum. Arrows at the right identify the three major molecular weight species of mouse keratins. Molecular weight markers are indicated in thousands at the left.

type, with no apparent decrease in pemphigoid Ag (Fig. 4, lanes a and b) and little or no increase in fluorescent staining for pemphigus Ag (Fig. 5d) or lectin binding (Fig. 6d), respectively. M RSV-infected cells showed a marked reduction in pemphigoid Ag expression (Fig. 4, lanes 5a and b) but little apparent change in the other two markers (data not shown). This pattern was consistent with these cells being blocked at a somewhat later stage of differentiation than that of the Abelson MuLV-infected cells. The pattern of epidermal marker expression by ST FeSV-infected cells at high levels of Ca^{2+} was consistent with their being blocked at an even later stage. They exhibited decreased levels of pemphigoid Ag (Fig. 4, lanes a and b) and increased pemphigus Ag expression (Fig. 5f) but no evidence of lectin binding (data not shown). Thus, our findings indicated that three different oncogenes of the tyrosine kinase family induced readily distinguishable alterations in the Ca^{2+}-induced differentiation program of BALB/MK-2 cells.

The expression of pemphigoid Ag by Kirsten and Moloney MSV-infected BALB/MK-2 cells did not appear to be greatly reduced in response to the high Ca^{2+} challenge (Fig. 4, lanes 2a and b and 3a and b, respectively). However, these cells exhibited high levels of pemphigus Ag and Ulex Europaeus I binding as represented by the Kirsten MSV-altered cells shown in Fig. 5h and 6f, respectively. Such alterations readily distinguished Kirsten and Moloney MSV-altered BALB/MK-2 cells from the other viral transformants. Table 3 summarizes the patterns of expression of epidermal differentiation markers in the uninfected and virus-altered BALB/MK-2 cells in the absence or presence of Ca^{2+} challenge.

DISCUSSION

The establishment of clonal BALB/MK-2 epidermal keratinocyte lines has made it possible to investigate alterations in growth and differentiation induced in cells of epithelial origin by the cell-derived *onc* genes of acute transforming retroviruses (34). The present studies demonstrate that several *onc* genes of the tyrosine kinase family supplant the EGF requirement for BALB/MK-2 cell proliferation. *src*, *abl*, and *fes* are related in their nucleotide sequences and code for proteins possessing tyrosine kinase activity. *mos*, a transforming gene whose product lacks detectable tyrosine kinase activity but possesses serine-threonine kinase activity (16), bears sequence similarities to the tyrosine kinase genes. Recent studies have shown that *ras* transforming genes, which bear no structural or known functional homology to the *src* family, also eliminate the EGF requirement of BALB/MK-2 cells (34). Thus, two major families of retroviral *onc* genes share the ability to replace the growth-promoting actions of an essential mitogen for BALB/MK-2 epithelial cell growth.

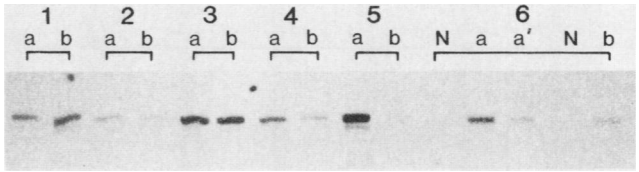
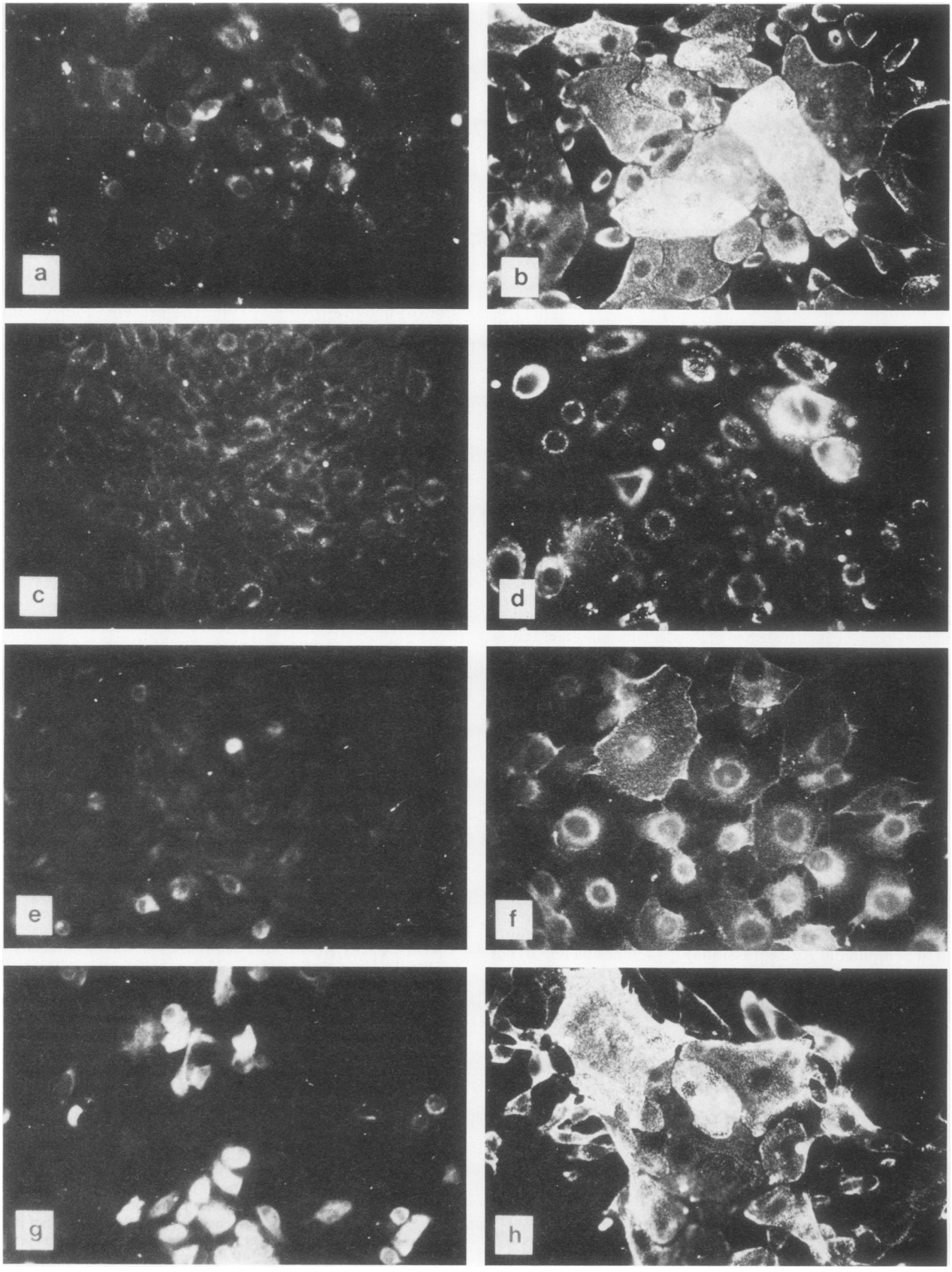


FIG. 4. Synthesis of bullous pemphigoid Ag by representative uninfected and virus-infected BALB/MK-2 cells. Cells were seeded at a concentration of 10^6 cells per 100-mm petri dish in low-calcium (0.05 mM) growth medium. When the cultures achieved semiconfluence, calcium chloride at a final concentration of 1.0 mM was added to half of the dishes. Three days later, the cultures were rinsed with PBS and incubated overnight in methionine-free MEM at the appropriate calcium concentration with 70 μCi of [^{35}S]methionine per ml. The cells were then solubilized in a Tris buffer (pH 7.4) containing 0.5% NP-40. After centrifugation, the supernatants were dialyzed against three changes of Tris buffer (pH 7.4) containing 0.3% NP-40. Samples containing equal counts per minute were preincubated with normal human serum for 2 h at 0°C. After removal of antibody-bound proteins by incubation with Pansorbin (Calbiochem-Behring), the samples were incubated overnight at 0°C with the appropriate antiserum. Immunoprecipitated proteins were isolated by incubation with Pansorbin and analyzed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (18). Similar results were obtained for different clones altered by the same virus. Cell extracts from [^{35}S]methionine-labeled Abelson MuLV-infected BALB/MK-2 (lane 1), Kirsten MSV-infected BALB/MK-2 (lane 2), Moloney MSV-infected BALB/MK-2 (lane 3), BALB/MK-2 (lane 4), M RSV-infected BALB/MK-2 (lane 5), and ST FeSV-infected BALB/MK-2 (lane 6) cells grown in either low (a) or high (b) levels of calcium were immunoprecipitated with two different antisera directed against bullous pemphigoid Ag (a and a') or normal human serum (N). The pemphigoid Ag migrated as a 220,000-dalton protein relative to the molecular size markers.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The mechanisms by which these transforming genes overcome the EGF requirement could involve their direct interaction at various steps in the cellular growth regulatory pathways normally responsive to EGF or through completely different pathways. Recent studies have shown that the *sis*-transforming gene encodes a polypeptide of human platelet-derived growth factor, a potent mitogen for connective tissue cells (9, 26). None of the presently analyzed oncogenes encodes a protein that resembles the known structure of EGF. A number of studies have documented the release of EGF-related factors from rodent fibroblasts transformed by various oncogenes (32). While we have not detected the release of such molecules from any of the virus-altered BALB/MK cells so far analyzed (B. Weissman, unpublished observations), systematic biological and molecular studies will be required to determine whether such factors play an indirect role in the growth alterations induced by oncogenes of the *src* and *ras* families. It should be noted that EGF does not block Ca^{2+}-induced terminal differentiation of BALB/MK-2 cells (34), while each of the *onc* genes analyzed allowed BALB/MK-2 cells to overcome this potent differentiation signal. Thus, it would be difficult to explain all of the actions of these oncogenes solely by their induction of EGF-like factors.

The effects of the tyrosine kinase-encoding oncogenes, *abl*, *src*, and *fes*, appeared to be consistent with their blocking the cells at progressively later steps in the keratinocyte differentiation pathway. The phenotype of *mos*- and *ras*- altered cells appeared to be at an even later stage based upon the stratification of cells observed in response to high levels of Ca^{2+} and the high level of expression of markers known to be associated with later stages of keratinocyte differentiation. There was also evidence of aberrations in the normal differentiation program induced by different oncogenes. For example, despite their expression of markers most consistent with a relatively early block in differentiation, M RSV-altered cells showed some stratification upon Ca^{2+} challenge. Conversely, *mos* and *ras* transformants did not show decreased levels of pemphigoid Ag upon stimulus with high levels of Ca^{2+} despite exhibiting other markers of more differentiated cells. Recently Yuspa and his co-workers (35) have also reported an unusual program of differentiation in Kirsten MSV-infected cultures of primary mouse epidermal keratinocytes. Thus, the blocks to terminal differentiation observed with different transforming genes may occur at specific and distinct steps in this pathway but may also reflect aberrant expression of differentiation markers by such cells.

Several of the viral oncogenes utilized were transduced in vivo by Moloney MuLV or resulted from in vitro constructions involving the Moloney MuLV genome. Thus, the different phenotypes of *abl*-, *ras*-, and *src*-altered keratinocytes are unlikely to be related to differences in their expression as directed by Moloney MuLV regulatory sequences. It will be of interest to determine whether differing levels of expression of the same oncogene may affect the transformed phenotype of the cells, particularly with respect to the stage at which differentiation is apparently blocked at high calcium concentration.

The EGF receptor possesses tyrosine kinase activity (7), as do a number of receptors for other growth factors and hormones (8). Recent studies have shown that *erb*-B, an *onc* gene related to members of the *src* family, bears striking homology in its predicted amino acid sequence to a large region of the EGF receptor (10). If, as is thought to be the case for *erb*-B, some other members of the *src* family represent activated forms of normal growth factor receptors, then their effects on BALB/MK-2 cells are not consistent with a simple activation of normal cellular proliferation. A powerful, sustained proliferative signal such as the constitutive expression of an activated growth factor receptor might explain the inability of BALB/MK-2 cells to respond to the Ca^{2+}-induced differentiation signal. However, this model would not readily account for blocks at apparently different points in the terminal differentiation pathway observed with different members of the *src* family. It will be of interest to investigate the effects of other oncogenes, particularly *erb*-B, on BALB/MK-2 cells. By this approach, it may be possible to identify, by their similar effects on the keratinocyte phenotype, those *onc* genes that function like the *erb*-B-EGF receptor at very early steps in the activation of cell proliferation.

There are some interesting parallels between the interactions of *onc* genes with keratinocytes and their effects on other cell types capable of undergoing differentiation. Acute retroviruses transform hematopoietic cells within a number of lineages under in vitro culture conditions in which normal hematopoietic cell proliferation is otherwise quite restricted by growth factor requirements (13, 21, 22, 28). Thus, retroviral *onc* genes also appear to overcome specific growth factor requirements of hematopoietic cells. After infection with transforming retroviruses, hematopoietic cells appear to be arrested at relatively specific stages in differentiation (21, 22, 28). This also seems to be the case with *src* infection of myeloblasts (11). While apparent blocks in differentiation or even dedifferentiation of more mature cell types have been associated with infection by some viral oncogenes (11), there have been rare reports of induction of differentiation as well (3, 19).

In hematopoietic cell culture systems, it has not been

FIG. 5. Immunofluorescence of pemphigus vulgaris Ag in uninfected and virus-infected BALB/MK-2 cells. Cells were seeded onto cover slips at a density of 3×10^4 cells per cover slip in low-calcium (0.05 mM) growth medium and allowed to reach ~90% confluence. Some cultures were then supplemented with calcium chloride at a final concentration of 1.0 mM. Three days later, the cover slips were stained directly with human antiserum against pemphigus vulgaris for 30 min at 37°C. The cover slips were rinsed three times with PBS and then were treated with fluorescein-conjugated goat anti-human immunoglobulin G (Huntington Laboratories) and incubated for 30 min at 37°C. After three rinses with PBS and one rinse with double-distilled water, the cover slips were inverted and mounted on glass slides with a solution of glycerol-PBS (1:1). Samples were viewed with a Zeiss photomicroscope fitted with a Ploem UV system. Typical photographic fields of uninfected BALB-MK-2 cells grown in low (a) or high (b) levels of calcium, Abelson MuLV-infectd BALB/MK-2 cells grown in low (c) or high (d) levels of calcium, ST FeSV-infected BALB/MK-2 cells grown in low (e) or high (f) levels of calcium, Kirsten MSV-infected BALB/MK-2 cells grown in low (g) or high (h) levels of calcium are shown (magnification, ×400). The pattern of immunofluorescence with Moloney MSV-altered cells was indistinguishable from that of Kirsten MSV-altered BALB/MK-2, while the pattern with M RSV-altered BALB/MK-2 cells was identical to that with Abelson MuLV-altered cells. The background immunofluorescence with normal human serum on the BALB/MK-2 cells in either low or high levels of Ca^{2+} was similar to that observed with the pemphigus vulgaris antiserum on the virus-altered BALB/MK-2 cells in low levels of Ca^{2+}.

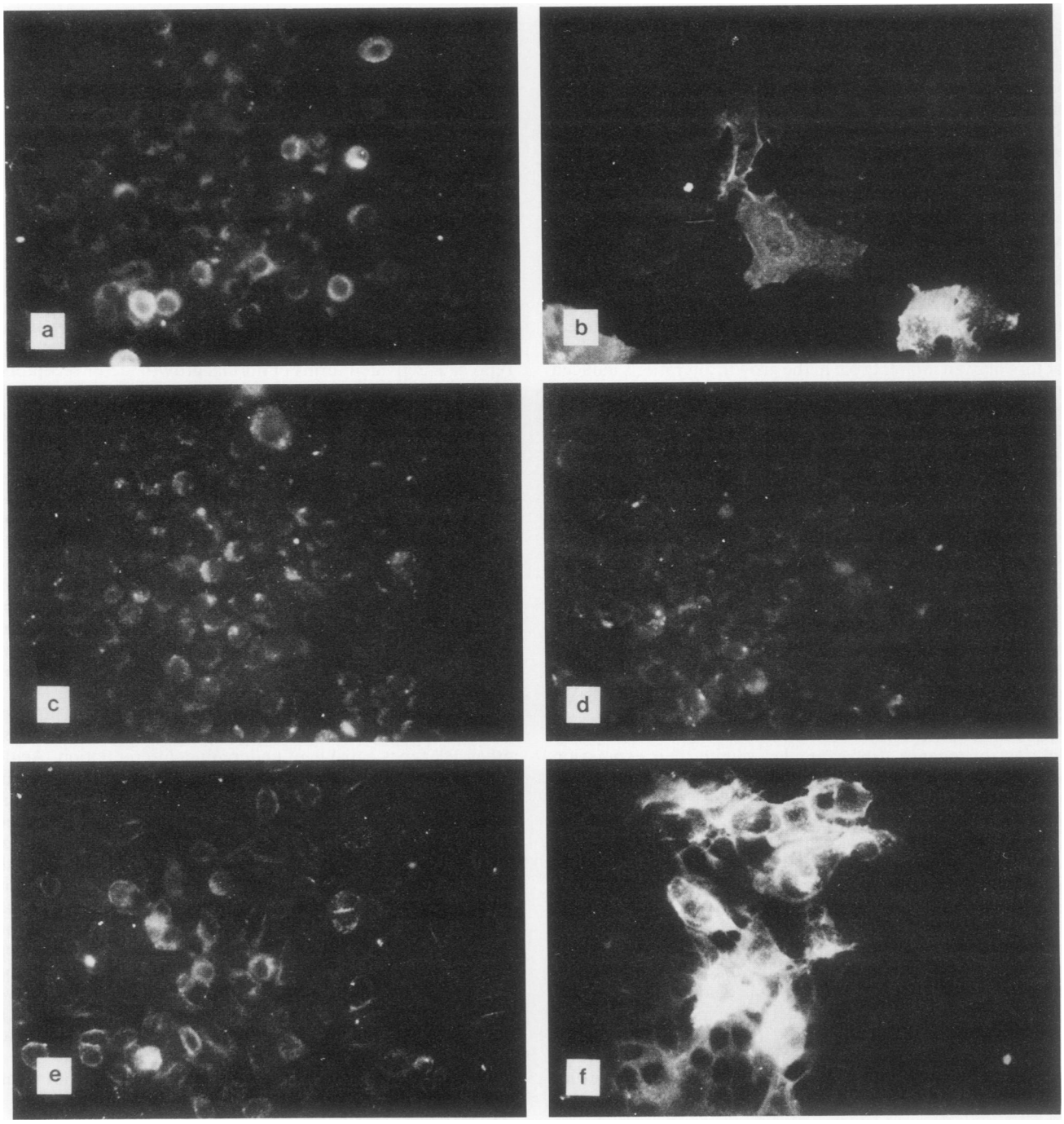

FIG. 6. Binding of fluorescein-conjugated Ulex Europaeus I lectin to uninfected and virus-infected BALB/MK-2 cells. Cells were seeded onto cover slips at a density of 3×10^4 cells per cover slip in low-calcium (0.05 mM) growth medium and allowed to reach ~90% confluence. Some cultures were then supplemented with calcium chloride to give a final concentration of 1.0 mM. Three days later, the cover slips were stained directly with fluoresceinated Ulex Europaeus I lectin (Vector Laboratories) for 30 min at room temperature. After three rinses with PBS and one rinse with double-distilled water, the cover slips were inverted and mounted on glass slides with a solution of glycerol-PBS (1:1) and viewed with a Zeiss photomicroscope fitted with a Ploem UV system. Typical microscopic fields were observed with BALB-MK-2 cells grown in low (a) or high (b) calcium levels, Abelson MuLV-infected BALB/MK-2 cells grown in low (c) or high (d) calcium levels, and Kirsten MSV-infected BALB/MK-2 cells grown in low (e) or high (f) calcium levels (magnification, ×400). The pattern of immunofluorescence observed with Moloney MSV-altered BALB/MK-2 cells was indistinguishable from that of Kirsten MSV-altered cells. Abelson MuLV-, M RSV-, and ST FeSV-altered cells were all negative for immunofluorescence at both low and high calcium concentration.

opossible to readily determine whether the specificity for transformation is due to infection and transformation of the same cell or whether infection of a precursor is associated with subsequent differentiation until arrest occurs at a particular stage. Our studies with BALB/MK-2 cells provide experimental proof of the latter model with respect to the interactions of oncogenes with the keratinocyte differentiation pathways. Infection of a common keratinocyte progen-

TABLE 3. Expression of epidermal differentiation markers by acute retrovirus-altered BALB/MK-2 cell lines

Treatment of cells (pseudotype)[a]	Presence of marker in cells grown in medium with[b]:					
	Low Ca^{2+}			High Ca^{2+}		
	Pemphigoid Ag	Pemphigus Ag	Ulex Europaeus I binding	Pemphigoid Ag	Pemphigus Ag	Ulex Europaeus I binding
Uninfected	High	Absent	Absent	Low	High	High
Abelson MuLV (M MuLV)						
Clone 1	High	Absent	Absent	High	Low[c]	Absent
Clone 2	High	Absent	Absent	High	Low[c]	Absent
M-RSV (M MuLV)						
Clone 1	High	Absent	Absent	Low	Low	Absent
Clone 2	High	Absent	Absent	Low	Low	Absent
ST FeSV (R MuLV)						
Clone 1	High	Absent	Absent	Low	High	Low
Clone 2	High	Absent	Absent	Low	High	Low
Moloney MSV (M MuLV)						
Clone 1	High	Low	Absent	High	High	High
Kirsten-MSV (R MuLV)						
Clone 1	High	Low	Absent	High	High	High
Clone 2	High	Low	Absent	High	High	High

[a] M MuLV, Moloney MuLV, R MuLV, Rauscher MuLV.
[b] The presence of pemphigoid Ag was determined by radioimmunoprecipitation and polyacrylamide gel electrophoresis of both uninfected and virus-infected BALB/MK-2 cells. Pemphigus Ag was detected by immunofluorescence of both uninfected and virus-infected BALB/MK-2 cells. Binding of Ulex Europaeus I lectin was determined by fluorescent microscopy techniques.
[c] Present but expressed in an aberrant pattern.

itor was associated with unmasking of specific blocks in the differentiation program upon Ca^{2+} challenge.

There is increasing evidence that some of the cellular genes transduced by acute transforming viruses can be activated as oncogenes directly in human tumor cells. Previous studies have further indicated that the terminal differentiation of human squamous cell carcinomas may be retarded (25), consistent with the alterations induced in BALB/MK cells by members of the *ras* or *src* gene families. The effects of oncogenes on the neoplastic potential of the parental BALB/MK-2 cell line remain to be determined. At the same time, it will be of interest to investigate how different oncogenes may interact to induce various manifestations of epithelial cell malignancy, possibly even the metastatic phenotype.

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