V-*Myc* Alters the Response of a Cloned Mouse Mammary Epithelial Cell Line to Lactogenic Hormones

Roland K. Ball, Andrew Ziemiecki, Cora A. Schönenberger, Ernst Reichmann, Shelagh M. S. Redmond, and Bernd Groner

Ludwig Institute for Cancer Research
Inselspital
3010 Bern, Switzerland

Several oncogenes have now been implicated in mammary carcinogenesis. We investigated the phenotypic effects of expressing three representative oncogenes in mammary epithelial cells. v-*myc* (coding for a nuclear protein), v-Ha-*ras* (a G-protein homologue) and v-*fgr* (a tyrosine kinase) genes were introduced into the nontumorigenic clone 14 of the mouse mammary epithelial cell line COMMA-1D. Their effects upon growth and differentiation were determined. Anchorage-independent growth was induced by all three oncogenes with low efficiency. v-Ha-*ras* and v-*fgr* induced tumorigenicity in nude mice. The effect of oncogenes upon parameters unique to mammary epithelial cells *in vitro* was assayed. Both v-*myc* and v-*fgr* abolished the ability of clone 14 to grow as three-dimensional branching structures in hydrated collagen gel. v-*fgr* completely and v-*myc* partially inhibited the expression of the epithelium specific cytokeratins. Clone 14 can be induced to produce the β-casein milk protein by the combination of the lactogenic hormones, dexamethasone, insulin, and PRL. Introduction of v-*myc* into clone 14 cells resulted in an estimated 50-fold increased induction of β-casein protein and at least a 60-fold increase in β-casein mRNA. The number of cells stained with anti-β casein antibodies also showed a 10-fold increase after v-*myc* introduction. This still required the synergistic action of all three lactogenic hormones. Thus v-*myc* can alter the normal response of mammary epithelial cells to lactogenic hormones. (Molecular Endocrinology 2: 133–142, 1988)

INTRODUCTION

At least four oncogenes have been implicated in the carcinogenesis of the mammary gland. Primary human breast carcinomas often show the loss of the c-Ha-*ras*-1 allele (1), amplification of *erb*B-2 (2), or amplification of c-*myc* (3). Overexpression of *erb*B-2 has also been seen in human mammary tumor cell lines (4). Transfection of DNA from human breast carcinoma cell lines has detected the activated Ha-*ras* (5), the amplified nonactivated N-*ras* (6), and the activated c-Ki-*ras* (6a) as transforming genes. Ha-*ras* is frequently activated in chemical carcinogen induced mammary carcinomas in rats (7) and mouse c-*myc* expressed from a MMTV LTR in transgenic mice was found to predispose the mice to mammary adenocarcinomas (8).

Oncogene effects have so far been mostly detected and studied by the transformation of fibroblasts. Little is known about their effect on mammary epithelial cells. Transformation of primary mammary epithelial cells *in vitro* by Simian Virus 40 (SV40) virus resulted in multiple phenotypes (9–11) presumably due to the heterogeneity of the breast epithelium (12, 13). SV40 virus infection of the uncloned mouse mammary epithelial cell line, NMuMG, did not result in tumors in syngeneic mice (14) whereas transfection of a human activated c-ras^H into NMuMG produced cells tumorigenic in nude mice (15).

We are studying the effects of known, cloned oncogenes on cultured mouse mammary epithelial cells. COMMA-1D cells were chosen as the most suitable mouse mammary epithelial acceptor cell line (16). This BALB/c cell line arose spontaneously after prolonged culture in low serum. It is nontumorigenic and has a normal karyotype. Its mammary epithelial nature was confirmed by expression of epithelial cytokeratins, the ability to repopulate the cleared mammary fat pad with ductal epithelium, and the induction of β casein milk protein by lactogenic hormones (16). For our studies we employed different classes of oncogenes: v-*myc* codes for a nuclear-located, immortalizing/transforming protein and v-Ha-*ras* codes for a plasma membrane located, transforming protein which can complement *myc* in the transformation of primary cells. v-*fgr* was used as a representative of the tyrosine kinase family of oncogenes. Their effects upon the growth properties and tissue-specific gene expression of the COMMA-1D cells was studied. Anchorage-independent growth and tumorigenicity in nude mice were induced. Three-dimensional growth in a collagen matrix was altered. v-*fgr* completely abolished expression of epithelium specific cytokeratins and v-*myc* increased the induction of the β-casein milk protein by lactogenic hormones.

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RESULTS

Characterization of Clones from the COMMA-1D Cell Line

The COMMA-1D cell line was found to become morphologically heterogeneous, tumorigenic, and anchorage independent upon passaging (17). For this reason it was subcloned by transfection of the hygromycin B resistance plasmid pY3 to recover cells retaining properties of normal mammary epithelial cells. Six clones showing widely different morphologies were analyzed further and their properties listed in Table 1.

Clone HC14 was chosen for our subsequent studies. It is the only clone which possesses most of the desired properties described for the original COMMA-1D cell line. HC14 is not tumorigenic in nude mice and does not show anchorage-independent growth. It retains the ability to form domes in culture and grows in a branching fashion in a hydrated collagen gel. Its epithelial origin was confirmed using monoclonal antibodies (18) which recognize human cytokeratin intermediate filaments usually expressed in simple epithelium. *In vivo* antibody LE61 stains epithelial cells and LP34 stains myoepithelial cells, thus the staining of HC14 with only LE61 would suggest that it is of the epithelial cell type. HC14 fails to express the Forssman antigen (detected by monoclonal antibody 117C9) present on all stromal cells in the mammary gland (19). HC14 expresses the 33A10 antigen present on highly differentiated mammary epithelial cells secreting (or about to secrete) milk proteins. This antigen represents a marker of a late differentiation stage (19). Like the COMMA-1D population, clone HC14 can be induced by lactogenic hormones to produce a low level of β-casein milk protein. Unlike the COMMA-1D population, no clone could repopulate the cleared mammary fat pad. Subsequently it was also found that additionally isolated clones did not retain the ability to repopulate the cleared fat pads and that clones that can be induced to produce casein as efficiently as COMMA-1D were very rare (data not shown and Medina, D., personal communication). Thus clone HC14 was chosen as the best acceptor cell available to study the effects of the introduction of oncogenes upon mammary epithelial cells. HC14 cells were used after a minimum of passaging (using frozen stocks of early passages) and parental cells which had been through an equivalent number of passages as the oncogene transformed cells were used as controls in the various assays for the phenotypic effects of the oncogenes.

Introduction of Oncogenes into HC14

The three oncogenes, v-*myc*, v-Ha-*ras*, and v-*fgr*, were now introduced into the HC14 cells either by transfection or by using recombinant murine retroviruses. The constructs used are shown in Fig. 1. v-*fgr* was introduced by cotransfection of pGR and pSV2-neo. The efficiency of cotransfection of HC14 cells and the level of *fgr* expression in epithelial cells required to obtain transformation are unknown. To select *fgr* expressing transformed cells we injected nude mice with a population of G418 resistant cells. This resulted in rapid tumor formation with a latency of 1 week. A G418-

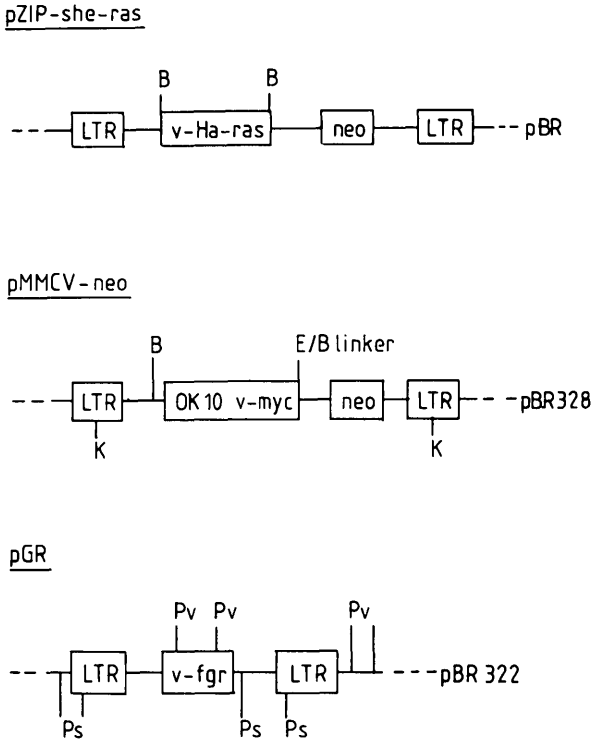

Fig. 1. Plasmids Used
B, *Bam*HI; E, *Eco*RI, K, *Kpn*I; Pv, *Pvu*II; Ps, *Pst*I.

Table 1. Phenotype of COMMA-1D Clones

Clone	2	3	14	17	21	31	COMMA-1D Population
Anchorage independence	–	–	–	–	–	–	+
Tumorigenic	+	+	–	+	+	–	+
Branching growth in collagen	–	–	+	+	–	+	+
Dome formation	–	+	+	–	–	+	+
Antihuman keratin 18 (LE61)	0	100%	100%	0	0	0	50%
Antihuman keratins (1, 8, 19)$^-$ (LP34)	0	100%	0	10%	90%	0	10%
Forssman antigen (117C9)	–	ND	–	ND	ND	–	ND
33A10 antigen	0	90%	80%	0	0	50%	90%
β-Casein induction	–	–	+	–	–	±	++

ND, Not determined.

selected tumor explant culture was used for further analysis. v-*myc* and v-Ha-*ras* were introduced by infection with recombinant retroviruses. For each construct used, pools of virus-infected cells were generated by combining in excess of 100 independent G418-resistant colonies.

Integration and Expression of Oncogenes

Integration of the proviral copies of the oncogenes was demonstrated by Southern blot analysis (Fig. 2). A 500 base pair (bp) *Pvu*II fragment from pGR spanning the *fgr* specific region detected a 500 bp PvuII fragment and a 2.7 kilobase (kb) *Pst*I fragment spanning both *fgr* and the first proviral LTR in genomic DNA from pGR transfected HC14. An 800 bp fragment from pVC-*ras* was used to detect the 800 bp BamHI insert of v-Ha-*ras* in DNA from ZIP-she-*ras* infected HC14. A 1.5 kb *Pst*I fragment of pMC-Pst (containing MC29 v-*myc*) was used to detect a 9 kb *Kpn*I fragment (*Kpn*I cuts both LTRs of the MMCV-neo virus), or a 2.8 kb *Bam*HI fragment (the OK10 v-*myc* insert at the *Bam*HI site) in DNA of MMCV-neo infected HC14.

The expression of the introduced oncogenes was confirmed. The p70 gag-actin-*fgr* fusion protein was demonstrated in pGR transfected HC14 by an *in vitro* kinase reaction upon immunoprecipitation with anti-gag antibodies (Fig. 3A). Phosphoamino acid analysis of the excised p70 band from pGR transfected HC14 showed mainly phosphotyrosine residues. The total phosphoamino acid content of *in vivo* labeled cells demonstrated an increase in total cellular phosphotyrosine due to the *fgr* tyrosine kinase (using two different extraction buffers). The phosphoserine, phosphothreonine, and phosphotyrosine content of HC14 was 86%, 13.4%, 0.6%, and of pGR transfected HC14, 86.8%, 10.4%, and 2.8% respectively. Thus v-*fgr* produced a 5-fold increase in total cellular phosphotyrosine.

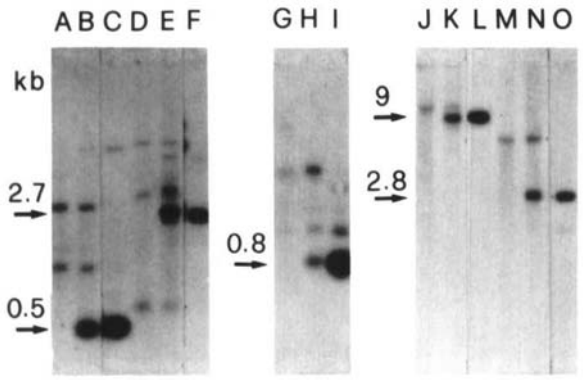

Fig. 2. Southern Blot Analysis of Oncogenes in HC14

Sixty picograms of digested plasmid DNA or 5 μg genomic DNA were applied per lane and hybridized to ^{32}P-labeled, nick-translated probes. DNA from HC14 (lanes A and D), pGR transfected HC14 (B and E) and pGR (C and F) were digested with *Pvu*II (A, B, C) or PstI (D, E, F) and hybridized with a *fgr* specific 500 bp *Pvu*II fragment from pGR. DNA from HC14 (G), ZIP-she-*ras* infected HC14 (H), and pZIP-she-*ras* (I) were digested with *Bam*HI and hybridized with a 800 bp *Bam*HI fragment of pVC-*ras* as a v-Ha-*ras* probe. DNA from HC14 (J and M), MMCV-neo infected HC14 (K and N) and pMMCV-neo (L and O) were digested with *Kpn*I (J, K, L) or *Bam*HI (M, N, O) and hybridized with a 1.5 kb *Pst*I fragment of pMC-Pst as a v-*myc* probe.

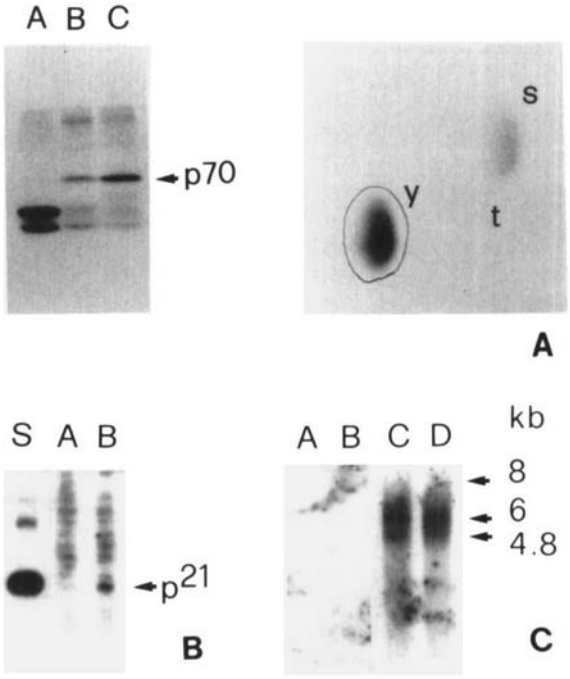

Fig. 3. Expression of Oncogenes in HC14

A, *In vitro* kinase assay of p70$^{gag\text{-}actin\text{-}fgr}$ in pGR-transfected HC14. *Left*, Lysates of HC14 (A), HC14 transfected with pGR (B) and NIH 3T3 transfected with pGR (C) were immunoprecipitated with anti-p15 (gag) antibodies, kinased *in vitro* with γ^{32}P-ATP, and resolved on 11% PAGE. *Right*, The p70$^{gag\text{-}actin\text{-}fgr}$ band detected in HC14 transfected with pGR was excised, hydrolysed, and resolved by two-dimensional electrophoresis with nonradioactive phosphoamino acid standards. s, Phosphoserine; t, phosphothreonine, y, phosphotyrosine. B, Western blot of p21 expressed in ZIP-she-*ras* infected HC14. Membrane preparations from HC14 infected with ZIP-she-*ras* (B) or the control ZIPneoSV(X) virus (A) were resolved on a 17% polyacrylamide gel, electroblotted onto nitrocellulose, reacted with rabbit anti-p21 antibodies, and then ^{125}I-labeled protein A and autoradiographed. Sixty nanograms of purified p21 protein from the human T24 cell line was applied as a standard (S). C, Northern blot analysis of v-*myc* mRNA expression. RNA was electrophoresed in denaturing agarose gels, transferred to nitrocellulose, and hybridized with a ^{32}P nick-translated 1.5 kb *Pst*I chicken v-*myc* probe from pMC-Pst. Five micrograms polyadenylated RNA per lane from the following cells. HC14 induced for 5 days in insulin (A), or insulin, dexamethasone, and PRL (B). MMCV-neo infected HC14 similarly induced with insulin (C), or insulin, dexamethasone, and PRL (D).

Membrane associated v-Ha-*ras* p21 protein was demonstrated in a Western blot analysis. Rabbit antibodies to human p21 detected the protein in the ZIP-she-*ras* infected HC14 and not in the control ZIPneoSV(X) infected cells (Fig. 3B). v-*myc* expression was confirmed by Northern blot analysis of RNA (Fig. 3C). The 4.8 kb spliced v-*myc* transcript (20) was detected by the v-*myc* specific probe in MMCV-neo infected HC14. Equal levels of expression were seen in

cells induced with insulin alone or induced with all three lactogenic hormones. The full-length genomic transcript at 8 kb was not seen. The origin of the 6 kb transcript is unknown but a similar additional transcript was seen when the parental MMCV virus was used in other cells (21). All three oncogenes introduced into HC14 are expressed.

Effects of Oncogenes upon Growth

The effects of the expression of the introduced oncogenes upon various growth parameters of HC14 cells were assayed. No change in morphology of the v-*myc* or v-*ras* containing cells was detected. The v-*fgr* containing cells consisted of a mixture of flat epithelial cells and altered rounded refractile cells. All oncogene-containing HC14 cells retained density inhibition of growth. The oncogene transformed cells were tested for anchorage independent growth in soft agar (Table 2). The oncogenes induced anchorage independence with low efficiency (less than 1%) after a latency of 3 weeks. The same constructs efficiently induced soft-agar growth in the NMuMG mammary epithelial cell line or NIH 3T3 fibroblasts (data not shown). The modest induction in anchorage independence could be due either to a low level of oncogene expression or to the fact that HC14 does not show anchorage independence even when transformed. It has been shown previously that anchorage independence does not correlate well with tumorigenicity of epithelial cells. Anchorage independence of SV40 transformed mouse mammary epithelial cells did not predict tumorigenicity (9) and tumorigenic natural human carcinoma cell lines showed very variable anchorage independence (22). Tumorigenicity of the oncogene-containing HC14 cells was tested sc in nude mice (Table 2). HC14 containing v-*fgr* or v-Ha-*ras* produced tumors with short latencies. HC14 or HC14 expressing v-*myc* did not cause tumors after 14 weeks of observation. Several other mouse mammary epithelial cell lines and clones (including the COMMA-1D clone HC31 described earlier) were, without exception, tumorigenic in nude mice after transformation with the same recombinant v-Ha-*ras* virus.

Three-dimensional branching growth in hydrated collagen provides an *in vitro* analog of ductal growth of the mammary epithelium in the mammary gland (23, 24). v-*myc* and v-*fgr* (but not v-Ha-*ras*) inhibited the branching growth of HC14 (Fig. 4) and so disturbed the growth behavior within an artificial basement membrane matrix.

Table 2. Anchorage Independence, Tumorigenicity, and Keratin Expression of HC14 with Introduced Oncogenes

HC14 Containing: -	Growth in Soft Agar[a]	Tumorigenicity[b]	% LE61$^+$ cells[c]
No construct	0	0/7	90%
ZIPneoSV(X) virus	0	0/2	80%
ZIP-she-*ras* virus	0.43	1/2 (3 w)	80%
MMCV-neo virus	0.16	0/2	20%
pGR	0.69	5/5 (1 w)	0%

[a] Percent cells forming colonies in 0.378% agar in growth medium (see *Materials and Methods*) at 3 weeks. Soft agar colonies ranged in size from about 20 cells per colony, upwards. 0 = Less than 0.002%.

[b] No. of tumors per no. of mice injected with tumor latency in weeks. Cells (10^6) injected sc in 2- to 3-week-old female Swiss nu/nu mice. Observed for 14 weeks.

[c] Indirect immunofluorescence using LE61 monoclonal anti-keratin antibody.

Effects of Oncogenes upon Differentiation

The effect of the oncogenes upon the differentiation of the HC14 mammary epithelial cells was examined. Expression of the 33A10 differentiation antigen in HC14 was not modified by introduction of oncogenes. Tissue specific cytokeratin expression and induction of milk proteins by lactogenic hormones were assayed. Expression of the keratin multigene family is developmentally regulated and different cell types within the mammary gland express different keratin polypeptides (12, 19). Alteration of expression of these epithelium specific intermediate filaments has been reported after transformation by SV40 of human keratinocytes (25) and human epidermal cells (26).

The effect of the introduction of oncogenes upon the cytokeratin expression of HC14 was examined. The number of cells stained by monoclonal anti-keratin antibody LE61 was determined using indirect immunofluorescence. Whereas over 80% of HC14 cells are stained by LE61, this number was reduced 4-fold in HC14 cells transformed by v-*myc* (Table 2). No v-*fgr* transformed cells could be stained at all. Immunoreactive cytokeratin was below detection in v-*fgr* transformed HC14 cells. Cytokeratin polypeptides were sub-

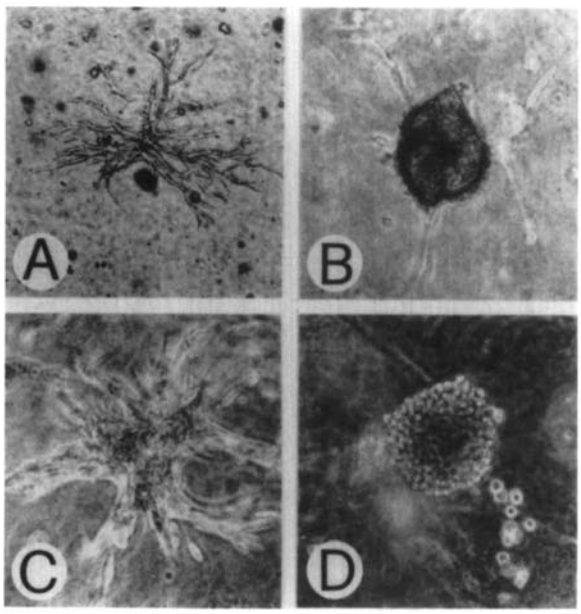

Fig. 4. Growth of HC14 in Collagen

A, HC14; B, MMCV-neo infected HC14; C, ZIP-she-*ras* infected HC14; D, pGR transfected HC14, after 2 weeks of culture in rat tail collagen in growth medium (*c.f. Materials and Methods*) as described previously (24).

sequently shown to be completely absent. The detergent insoluble cytoskeletal proteins present in HC14 before and after transformation by v-*fgr* were analysed by two-dimensional electrophoresis (Fig. 5) performed as described (27). All the cytokeratins found in HC14 were lost upon v-*fgr* transformation and the one remaining spot, staining with increased intensity, is most likely vimentin which is expressed in many mammary epithelial cell lines in culture (27). This provides another example of the alteration of tissue specific keratin expression in epithelial cells by oncogenes.

One form of differentiation of mammary epithelial cells is represented by the secretion of milk proteins by alveolar cells during lactation. The COMMA-1D cell line could be induced by culturing with the combination of lactogenic hormones comprising dexamethasone, insulin, and PRL to produce the β-casein milk protein. We used an indirect immunofluorescence assay with polyclonal rabbit anti mouse casein antisera to detect α, β, and γ caseins in mouse milk (28). Approximately 10% of COMMA-1D, 1% of HC14, and 0.1% of HC31 cells were stained after 5 days of hormone induction. No cells were stained when they were cultured with insulin in the medium only. We have confirmed that the percentage of midpregnant mouse mammary epithelial cells that can be stained for β-casein drops from 40% to 10% after culture *in vitro* on plastic (29). Only a low percentage of HC14 cells was detected as making β-casein, as the *in vitro* culture conditions employed are unable to induce all potentially responsive cells and some cells would be below the threshold of detection in the immunofluorescence assay because of the low level of β-casein produced.

The casein polypeptides produced by COMMA-1D were examined by Western blot analysis (Fig. 6). The estimated mol wts of the mouse milk caseins are:—α-1 [43 kilodaltons (kDa)], α-2 (42 kDa), β (31 kDa), and γ (27 kDa). Only β-casein was detected, no α or γ-casein. The identity of the 38 kDa band detected in cell extracts (running in the position between α- and β-casein) is unknown and may be due to a cross-reaction of the antisera or nonspecific binding. β-Casein is not secreted into the medium by HC14 (lanes D and E) or

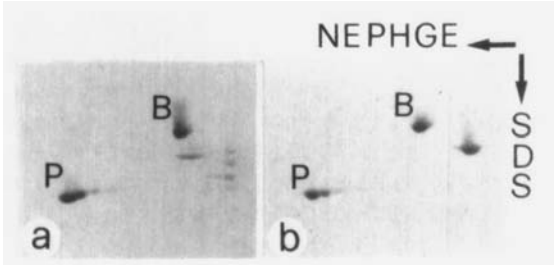

Fig. 5. Effect of v-*fgr* upon Cytokeratin Expression in HC14

Cytoskeletal proteins in detergent insoluble extracts from HC14 cells (a), or pGR transfected HC14 cells (b) were analyzed by two-dimensional electrophoresis as described (27). Cells were grown to confluence in growth medium. Gels were stained with Coomassie blue. Markers added: B, BSA (68 kDa), P, phosphoglycerate kinase (47 kDa). Nonequilibrium pH gradient gel electrophoresis as first dimension. SDS-polyacrylamide gel electrophoresis as the second dimension.

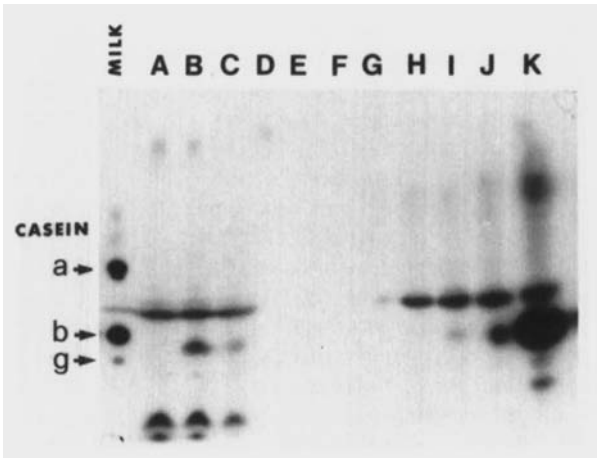

Fig. 6. Western Blot of Casein Produced by HC14 under Various Culture Conditions

Cell extracts were used from HC14 and 3T3-L1 adipocytes cocultured (A, B, C), HC14 only (H and I) or COMMA-1D (J and K). Trichloroacetic-acid precipitated culture supernatant was used from HC14 (D and E) or COMMA-1D (F and G). Cultures were induced for 5 days with insulin alone as a control (A, D, F, H, J) otherwise with dexamethasone, insulin, and PRL. Lane C represents induction with hydrocortisone, insulin, PRL, and aldosterone. Ten microliters of a 10^{-4} dilution of mouse milk was used as a standard for caseins (MILK). The locations of α-1 and α-2 casein (a), β-casein (b), and γ-casein (g) in mouse milk are indicated. Samples were electrophoresed in an 11% polyacrylamide gel, electroblotted, and reacted with rabbit anti-casein antisera and then ^{125}I-protein A.

COMMA-1D (lanes F and G) when cultured on plastic Petri dishes. The β-casein is found only in the cell extract. Failure of mammary epithelial cells to secrete casein when grown on a plastic substrate has been reported previously (30). The β-casein present in the cell extract has a slightly faster migration (with a mol wt of 28.5 K) than secreted milk β-casein. This is most likely due to a lack of phosphorylation (31, 32). We confirmed the identity of the smaller intracellular β-casein protein by showing that it was recognized by a monospecific rabbit antiserum prepared against β-casein purified from mouse milk. HC14 (lanes H and I) produce approximately 80-fold less β-casein than the COMMA-1D population (lanes J and K). Unlike primary cultures of mouse mammary epithelial cells (33), HC14 does not show increased induction of β-casein (lanes A, B, and C) upon coculture with converted 3T3-L1 adipocytes when compared to culture on plastic (lanes H and I). The protein content of the samples had been normalized within two groups, namely, A to C (the HC14/adipocyte cocultures) and H to K (cultures of epithelial cells alone). The β-casein protein signal did not significantly differ from the estimated relative HC14 protein content of the extracts from the cocultures and cultures of HC14 alone. β-Casein induction using the lactogenic hormone mixture originally described for COMMA-1D (16), namely, hydrocortisone, insulin, PRL and aldosterone (lane C) was not superior to the combination of dexamethasone, insulin, and PRL (lane B) used throughout these experiments. The nature of the

high mol wt protein induced by lactogenic hormones in COMMA-1D is unknown. The bands of smaller size than β-casein could be proteolytic products of β-casein or an immunological cross-reaction with other casein species of smaller mol wt.

The effect of the introduced oncogenes upon the induction of β-casein was examined. One percent of HC14 could be stained with anti-casein antibodies after 5 days of lactogenic hormone stimulation. No cells were stained which contained the ZIPneoSV(X) control virus, v-Ha-*ras* or v-*fgr*. However the frequency of casein producing cells was elevated 10-fold in the HC14 cells containing v-*myc*. These results were paralleled by the intracellular β-casein protein concentration determined in a Western blot analysis.

The response of the v-*myc* transformed cells was analyzed further. Lactogenic hormone induction of β-casein mRNA in HC14 was not detectable by Northern blot analysis (Fig. 7). An equal, very low level of β-casein mRNA was present in cultures with or without lactogenic hormones. Lactogenic hormone treatment of v-*myc* transformed HC14 gave a strong induction of the β-casein mRNA transcript with a size of 1.5 kb estimated using the ribosomal RNAs as markers. A 1.45 kb mouse β-casein mRNA transcript was previously reported (34). We estimated that at least 60-fold more β-casein mRNA was present in lactogenic hormone induced HC14 cells transformed by v-*myc*, compared to the original HC14 cells (lane A compared to lane C). To examine the effect of v-*myc* on β-casein protein induction HC14 cells were infected with either the MMCV-neo virus or the MV4-neo virus which is the control retroviral vector lacking only the v-*myc* insert (20). In both cases high titer stocks containing MoMuLV helper virus were used to generate pools of in excess of 2000 G418-resistant colonies. Western blot analysis demonstrated that casein induction was below detection in the MV4-neo infected HC14 whereas an estimated 50-fold more β-casein protein was induced in the v-*myc* transformed cells compared to HC14 (lane F compared to lane B, Fig. 7).

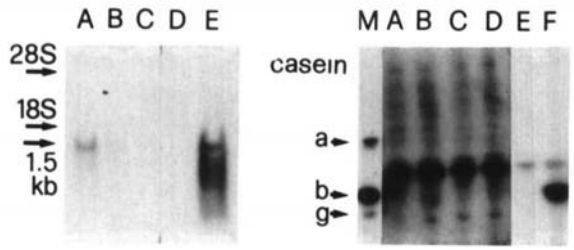

Fig. 7. Analysis of β-Casein mRNA and Protein Induced in HC14 after v-*myc* Transformation

Left, In a Northern blot analysis of β-casein mRNA expression, RNA was hybridized with a ^{32}P-nick translated mouse β-casein probe (a 0.5 kb *Eco*RI fragment of pA200 comprising most of the partial cDNA insert). Five micrograms of polyadenylated RNA were used from the following cells. MMCV-neo infected HC14 cells induced for 5 days with insulin, dexamethasone, and PRL (A) or insulin alone (B). HC14 cells induced with insulin, dexamethasone and PRL (C) or insulin alone (D). Five micrograms total RNA from lactating mouse mammary gland were used as a control (E). *Right*, β-casein protein detected by Western blotting of extracts from HC14 (A and B), MV4-neo infected HC14 (C and D), MMCV-neo infected HC14 (E and F). Cultures were induced for 5 days with insulin and dexamethasone (A, C, E) or insulin, dexamethasone, and PRL (B, D, F). The casein standards in mouse milk (M) are indicated as in Fig. 6. The autoradiograph was exposed 8-fold longer for lanes A–D.

One possible effect of v-*myc* could be an altered hormonal dependence of casein induction. All possible combinations of the three lactogenic hormones were tested for β-casein induction. All three (dexamethasone, insulin, and PRL) were required for the induction of β-casein-producing cells (detected by immunofluorescence) in COMMA-1D, HC14, and also HC14 transformed by v-*myc*. Thus v-*myc* did not alter the hormonal requirements for β-casein induction.

DISCUSSION

Several oncogenes have now been implicated in human breast cancer but little is known about the effect of oncogenes upon the growth and differentiation of mammary epithelial cells. We chose to use the COMMA-1D mammary epithelial cell line as the most suitable candidate for a model system. We isolated a clone (HC14) which retained most of the desired properties of normal mammary epithelial cells. They are nontumorigenic, do not show anchorage independent growth in soft agar, form domes, grow with a branching morphology in hydrated collagen, express cytokeratin, and can be induced by lactogenic hormones to differentiate further to produce the β-casein milk protein. The actual mechanism by which *ras*, *myc*, and *erb*B-2 (1–6) contribute to the carcinogenesis of the mammary gland is unknown. We chose to use viral oncogenes with proven transforming potential though their mode of action may differ slightly from cellular oncogenes involved in mammary carcinogenesis. Representatives of three different families of oncogenes were chosen namely: v-*myc* (coding for a nuclear protein), v-Ha-*ras* (a homologue of G-proteins), and v-*fgr* (a tyrosine kinase) to study the effects of oncogenes upon the growth and differentiation properties unique to mammary epithelial cells.

The v-*fgr* transformed HC14 cells were tumorigenic, anchorage independent, had altered growth in collagen, and had lost all cytokeratin expression. This is a unique example of the complete loss of cytokeratin expression upon transformation. The effect of v-Ha-*ras* on HC14 was less pronounced, producing tumorigenicity, anchorage independence but no effect upon growth in collagen or cytokeratin expression. The literature contains no previous examination of the biological effects of v-*fgr* on any cell type. The v-*fgr* transformed NIH 3T3 cells used in this study are tumorigenic (Kozma, S., unpublished). Activated human c-Ha-*ras* alone has been shown to confer tumorigenicity upon the NMuMG mouse mammary epithelial cell line (15) and upon primary rabbit mammary epithelial cells but only in conjunction with SV40 (11). If instead, *src* is considered as a representative prototype of the tyrosine kinase family

of oncogenes then both *ras* and *src* have been found to be able to modify the differentiation of other cell types. Both *ras* and *src* cause a block in the differentiation of mouse epidermal keratinocytes (35). *Src* was also able to block the differentiation of mouse preadipocytes (36), quail myogenic cells (37), and chicken chondroblasts (38), but in another cell type, neuronal cells, *ras* and *src* were found to have the opposite effect of promoting differentiation (39, 40).

An unexpected result was our finding that v-*myc* modulates the response of HC14 to lactogenic hormones, increasing the β-casein induced almost up to the amount produced by the original COMMA-1D cell line. The expression of v-*myc* in clone HC14 was shown to increase the induction of β-casein protein approximately 50-fold and mRNA at least 60-fold. The number of cells stained by indirect immunofluorescence with anti-casein antibodies was increased 10-fold. Cells containing v-*myc* still required the synergistic action of all three lactogenic hormones for β-casein induction. The effect could be due to an increase in the number of cells being hormone responsive. Casein gene expression is known to be controlled by the synergistic action of lactogenic hormones (41) increasing both transcription rate and mRNA stability (42). The response of mammary epithelial cells *in vitro* is also highly dependent upon the matrix used in tissue culture. A collagen matrix is superior to plastic (16, 30) but coculture with adipocytes (33) most effectively provides the necessary requirements for matrix and cell-cell interactions *in vitro*. However, the lactogenic hormone induction of β-casein in clone HC14 was not improved by coculture with adipocytes (in contrast to primary cultures of epithelial cells from midpregnant mouse mammary glands). v-*myc* could be acting by substituting for some tissue organization required specifically for milk protein expression. v-*myc* did not abrogate the requirement for any of the three lactogenic hormones as all were still required for a maximal synergistic response.

These results suggest that the expression of v-*myc* promotes the expression of the β-casein gene which is associated with mammary epithelium differentiation. The 4-fold reduction in the number of HC14 cells staining for cytokeratin after v-*myc* transformation would not be incompatible with an increase in differentiated state. The differentiation of mammary epithelial cells into alveolar cells secreting (or about to secrete) milk is naturally accompanied by the loss of cytokeratin expression. This has been shown in the mouse (19) and rat (12). Thus loss of cytokeratin expression and milk production are both markers of terminal differentiation. Our results differ from reports in which exogenously introduced v-*myc* blocked differentiation of quail myogenic cells (37) and exogenous c-*myc* blocked mouse erythroleukaemia cell differentiation (43, 44). Conversely, in almost all cases of induced cell differentiation examined so far, endogenous c-*myc* expression is suppressed (45, 46) and introduction of constitutively expressed v-*myc* has been shown to suppress transcription of endogenous c-*myc* (47). However, *myc* expression and differentiation are not always mutually exclusive for c-*myc* did not block chondroblast differentiation (38). c-*myc* can actually be re-induced in terminally differentiated myotubes (48) which remain differentiated.

Mammary epithelium is composed of several different epithelial cell types and the results described above are based on one cultured cell clone. Our results might not be entirely representative for the action of oncogenes in an *in vivo* situation in the mammary gland. This has recently been studied by C. A. Schoenenberger (48a) who directed the expression of a mouse c-*myc* transgene to the differentiating mammary epithelium of mice using the 5′-regulatory region of the murine whey acidic protein gene. This resulted in an 80% incidence of mammary carcinomas. The tumors express not only the Wap-*myc* transgene but also the endogenous Wap and β-casein genes. The expression of the milk protein genes becomes independent of the lactogenic hormone stimulation and persists even in transplanted nude mouse tumors. Here the effect of murine c-*myc* (as opposed to our use of v-*myc*) was tested in the mammary gland *in vivo*. Again a modification of the lactogenic hormone induction of milk proteins was seen, but in this case hormone independence resulted instead of an increased induction.

Targets of action of *myc* are not yet determined but there are now many precedents for a modification by *myc* of the cellular response to hormones. Introduction of v-*myc* (49) or constitutively expressed c-*myc* (50–52) resulted in hypersensitivity in the response to growth factors and v-*myc* could completely abrogate the IL-3 dependence of an early myeloid cell line (47). Thus *myc* is involved in the control of proliferation and differentiation and in the case of mammary epithelial cells this involves at least a change in responsiveness to lactogenic hormones.

MATERIALS AND METHODS

Cell Lines

COMMA-1D cells (16) were obtained from D. Medina (Houston TX) at passage 9 and maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 50 μg/ml gentamycin, 5 μg/ml insulin, and 10 ng/ml epidermal growth factor (growth medium) in an atmosphere of 5% CO_2-95% air. This medium was used for all cultures except for treatment with lactogenic hormones (*cf.* Casein induction). 3T3-L1 (a gift from H. Green, Cambridge, MA) was propagated and converted to adipocytes as described (33). For coculture, 10^7 HC14 cells (equivalent to 50% of confluence) were plated onto the converted adipocytes in 9-cm dishes 1 day before hormone induction of casein.

Plasmids

Antibiotic resistance plasmids used were pSV2-neo (53) conferring G418 resistance and pY3 (54) conferring hygromycin B resistance. Viral oncogenes were obtained as follows. The coding sequence of viral Ha-*ras* (55) was cut out as a 800 bp *Bgl*I-*Pst*I fragment, converted to *Bam*HI sites and cloned in pUC 18 to give pVC-*ras*. This *Bam*HI fragment was cloned into the pZIPneoSV(X) plasmid (56) with the *ras* sequence in the

same transcriptional orientation as the LTRs and neo gene to generate pZIP-she-*ras* (56a). v-*fgr* was present within a proviral copy of the Gardner-Rasheed feline sarcoma virus (57, 58) cloned in pBR322 at the *Hind*III site giving pGR (kindly provided by K. C. Robbins, Bethesda, MD). pMMCV-neo (20, 21) contained the v-*myc* oncogene from the avian retrovirus OK10, inserted upstream of a bacterial neo gene with a thymidine kinase promoter, all within a MLV based retrovirus vector pMV4-neo (both kindly provided by B. Vennström, Heidelberg, West Germany). pMC-Pst contains a 1.5 kb *Pst*I v-*myc* specific fragment from the MC29 virus (59). pA200, a gift from L. Hennighausen (Bethesda, MD) (34; unpublished), contains a partial cDNA clone equivalent to the carboxyl-terminal half of the mouse β-casein protein.

Nucleic Acid Analysis

Standard protocols were used for isolation of DNA, RNA, poly(A)$^+$ RNA (using oligo(dT) cellulose), nick translation, and RNA blotting analysis of glyoxal denatured RNA onto nitrocellulose after electrophoresis (60). DNA blotting analysis with Gene Screen Plus followed the manufacturer's instructions. DNA filters were prehybridized and hybridized overnight in 12.5% dextran sulfate, 1.25% sodium dodecyl sulfate (SDS) at 65 C and washed with 0.1 × SSC, 1% SDS at 65 C. RNA filters were hybridized in 4 × SSC, 50% (vol/vol) formamide, 5 × Denhardt's solution, 0.2% SDS, 0.3 mg/ml yeast transfer RNA, 0.1% sodium pyrophosphate, at 42 C overnight and washed in 0.5 × SSC, 0.2% SDS at 42 C.

Virus Production, Infection, and Transfection

Virus stock, free of helper virus, was made by transfecting ψ-2 cells (61) with the recombinant retroviral constructs. Culture supernatant of transfected cells had a virus titer of 10^3 to 10^4 G418-resistant NIH 3T3 colony-forming units per ml. Virus-containing culture supernatant was filtered through 0.45-μm filters, polybrene was added to 8 μg/ml, and then adsorbed to cell cultures for 2 h. Cells (5×10^5)/9-cm dish, plated 1 day before, were infected and 200 μg G418/ml were added 48 h later. Cells were transfected according to the protocol of Wigler *et al.* (62) using the calcium phosphate precipitation technique (63). Cells (5×10^5)/9-cm dish, plated 1 day before, were transfected with a 10:1 ratio of construct-drug resistance marker adding NIH 3T3 carrier DNA up to a total of 20 μg/plate. Drug selection (200 μg/ml G418 or hygromycin B) was applied 48 hr later without splitting the cultures.

Indirect Immunofluorescence

Cultures on plastic Petri dishes were washed with PBS and fixed for 3 min with acetone-methanol, 1:1 at −20 C, air-dried, and stored at −20 C. To detect the Forssman antigen with monoclonal 117C9 the cultures were fixed with 2.5% paraformaldehyde pH 7.3 in PBS for 15 min at 4 C, washed with PBS, blocked with fetal calf serum (FCS)-containing culture medium for 15 min at 4 C, PBS washed, and stored at −20 C. Five microliters of first antibody was incubated for 30 min at 37 C, washed with PBS, followed by 15 μl second antibody as before, and mounted with Mowiol (Hoechst, Frankfurt, West Germany) containing 50 mg/ml 1,4-diazabicyclo[2.2.2]octane as an antiquenching agent. Monoclonal antibodies were used as undiluted culture supernatant. Rabbit antimouse casein and control rabbit serum were diluted 1:80. Fluorescein-isothiocyanate conjugated antiimmunoglobulin second antibodies were diluted 1:40. Mouse monoclonal antihuman keratin antibodies were obtained from B. Lane (18): LE61 (anti-18) and LP34 (anti-many keratins but not 1,8,19). Sonnenberg *et al.* (19) supplied the rat monoclonal antibodies 117C9 (anti-Forssman antigen) and 33A10. Rabbit antimouse casein was supplied by Durban *et al.* (28). R. Sweet (Philadelphia, PA) supplied rabbit antisera against human *ras* p21.

Kinase Assays and Phosphoamino Acid Analysis

These were performed as described (64) with the following modifications. Cells in log growth were lysed in buffer containing 100 μM Na_3VO_4 and immunoprecipitated with antiserum (containing anti-p15 antibodies) prepared against disrupted feline leukemia virus.

Casein Induction

Cells were grown on plastic Petri dishes in growth medium for an additional 1–2 days after reaching confluence. Hormone induction medium was then added consisting of growth medium omitting epidermal growth factor to promote differentiation (65) with the addition of 10^{-6} M dexamethasone, 5 μg/ml insulin (bovine), and 5 μg/ml PRL (ovine). Cultures were induced for 4–5 days with one medium change. This protocol was also used for lactogenic hormone treatment of cells before isolation of mRNA for v-*myc* and β-casein analysis.

Protein Blot Analysis

To detect casein in cell extracts a 9-cm Petri dish culture was washed with PBS and lysed directly in 0.5 ml electrophoresis sample buffer (0.125M Tris·HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol) shearing the DNA through a 25-gauge needle and stored at −20 C. For secreted casein, 300 μl culture supernatant were precipitated with 20% trichloroacetic acid (final concentration) and dissolved in 100 μl sample buffer. Membrane-associated v-Ha-*ras* p21 was isolated, electroblotted, and detected as described (66). Five to 30 μl extract were applied per lane, normalizing the total protein content of extracts within each experiment. Autoradiograms from Western and Northern blots were quantified by laser densitometry.

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Address requests for reprints to: Roland K. Ball, Ludwig Institute for Cancer Research, Inselspital, 3010 Bern, Switzerland.

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