The EMBO Journal vol.3 no.13 pp.3223 – 3229, 1984

Transformation of mammalian fibroblasts and macrophages *in vitro* by a murine retrovirus encoding an avian v-*myc* oncogene

Björn Vennström, Patricia Kahn, Becky Adkins, Paula Enrietto[1], Michael J. Hayman, Thomas Graf and Paul Luciw[2]

European Molecular Biology Laboratory, Meyerhofstr. 1, 6900 Heidelberg, FRG, [1]Imperial Cancer Research Fund Laboratories, St. Bartholomews Hospital, Dominion House, London EC1A 7BE, UK, and [2]Chiron Corporation Inc., 4560 Horton, Emeryville, CA 94608, USA

Communicated by L. Philipson

A murine retrovirus which expresses the avian v-*myc*^{OK10} oncogene was constructed. The virus, denoted MMCV, readily transforms fibroblasts of established lines, such as mouse NIH/3T3 and rat 208F cells, to anchorage-independent growth in agarose. The virus also transforms primary mouse cells: (i) virus-infected macrophages are induced to form large colonies in semi-solid media, and can easily be expanded into mass cultures; (ii) MMCV-infected fibroblastic cells from mouse limb buds undergo morphological transformation and grow in semi-solid medium. MMCV thus transforms both mouse fibroblastic cells and macrophages *in vitro*, in a fashion similar to the v-*myc*-containing avian viruses in chicken cells. The possibility of introducing a transforming *myc* gene into mammalian cells by virus infection provides a novel approach for studying the mechanism of *myc* transformation in cells from many lineages.

Key words: cell transformation/oncogenes/*myc*

Introduction

Much evidence indicates that the *myc* gene is involved in the induction of a variety of neoplasms in different animal species. However, only in avian systems has it so far been possible to demonstrate rigorously that v-*myc* can cause neoplasia. In chickens, the *myc*-containing defective leukemia viruses (DLVs) MC29, CMII, MH2 and OK10 induce predominantly myelocytomatosis, endotheliomas or carcinomas (Graf and Beug, 1978). In addition, a deletion mutant of MC29 which has probably regained c-*myc* sequences causes predominantly B- and T-lymphoid cell tumors (Enrietto *et al.*, 1983). Infection of avian cells *in vitro* with the *myc*-containing DLVs has shown that these viruses transform primary avian fibroblasts and macrophages (Graf, 1973; Langlois *et al.*, 1969; Beug *et al.*, 1979; Gazzolo *et al.*, 1979). However, the transformed fibroblasts are not tumorigenic in nude mice (Palmieri *et al.*, 1983) and appear not to induce tumors in syngeneic chicks unless the cells produce infectious virus (Royer-Pokora *et al.*, 1978), suggesting that the induction of tumors by *myc* viruses requires continual viral recruitment of uninfected cells.

In mammals, there is only indirect evidence for the role of the *myc* gene in tumorigenesis. There are multiple copies of c-*myc* in cell lines established from a neuroendocrine colon carcinoma, in a promyelocytic leukemia cell line (HL60) and in small cell lung cancer (Alitalo *et al.*, 1983b; Little *et al.*, 1983; Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982). In addition, chromosomal rearrangements involving the c-*myc* locus appear to be associated with lymphomas in at least two mammalian species (Klein, 1983).

Studies on the mechanism of *myc* transformation of mammalian cells have been hampered by the lack of efficient *in vitro* transformation systems with *myc*. Transformation of mammalian cells with MC29, by virus infection or by transfection, occurs only at very low frequencies (Quade, 1979; Copeland and Cooper, 1980). Transfection of NIH/3T3 cells with DNA from tumor cells containing activated c-*myc* genes (activated by promoter insertion, translocation or gene amplification) have so far failed to reveal a transforming activity associated with c-*myc*. Instead, other oncogenes, which are presumably also activated in these tumor cells, have been detected in the NIH/3T3 transfection assays (Cooper and Neiman, 1980; Murray *et al.*, 1983, and personal communications). This, together with the recent data of Land *et al.* (1983) on the cooperativity of *myc* and *ras* in the tumorigenic conversion of primary mouse cells, have been taken to suggest that *myc* by itself is unable to transform mammalian cells *in vitro*, or to induce tumors, and that at least one other oncogene must also be involved in tumorigenesis.

To study the effects of *myc* in mammalian cells, we constructed a murine transducing retrovirus which expresses an avian v-*myc* oncogene. This virus, MMCV, transforms rodent fibroblasts and macrophages *in vitro*, and it can probably be used to study the effects of *myc* in other cell types as well.

Results

Strategy for expressing a myc oncogene in mammalian cells

To introduce efficiently a known transforming *myc* gene into different mammalian cells, including those refractory to transfection, we chose to insert *myc* into the genome of a murine retrovirus, thus facilitating an efficient and stable integration and expression of the gene in virus-susceptible cells. The retrovirus vector used, pMV-4, was cloned into the bacterial plasmid pBR328 and it contains 5′ and 3′ murine retroviral long terminal repeats (LTRs), derived from Moloney murine leukemia virus (MoMuLV) and Harvey sarcoma virus, respectively, a MoMuLV splice donor site and sequences required for packaging viral RNA into virus particles. The v-*myc* oncogene of the avian retrovirus OK10 was inserted into this vector. v-*myc*^{OK10} is expressed by OK10 *via* a subgenomic mRNA (Chiswell *et al.*, 1981; Saule *et al.*,

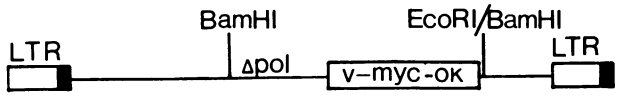

Fig. 1. Proviral structure of MMCV DNA. The murine retroviral sequences are derived from molecular clones of MoMuLV and Harvey sarcoma virus DNAs, and the avian retroviral sequences are from OK10 DNA as indicated. Restriction endonuclease cleavage sites relevant for this communication are indicated. LTR: long terminal repeat.

1982) as a *myc* protein with no *gag* sequences, which is similar to the *myc* protein in bursal lymphoma cells with LTR-activated c-*myc* genes (Pachl *et al.*, 1983; Hann *et al.*, 1983; Alitalo *et al.*, 1983a). We expected that the hybrid retroviral genome (Figure 1), designated murine *myc*-containing virus (MMCV), would express v-*myc via* a subgenomic mRNA with MoMuLV leader sequences spliced onto the acceptor site preceding v-*myc*^{OK10}. With this strategy we wished to avoid potential difficulties arising from introducing the *gag-myc* hybrid gene of MC29, which encodes a splice donor site within *gag* (Hackett *et al.*, 1982) and which also may contain sequences required for the packaging of avian retroviral RNA into avian retrovirus particles (Schwarz *et al.*, 1983).

Table I. Transfection of NIH/3T3 cells

DNA	Foci per μg DNA
MMCV	76
M-*src*	>400
MoMuLV	0

NIH/3T3 cells were transfected as described in Materials and methods. MMCV foci were counted 3–4 weeks and M-*src* foci 2 weeks after transfection.

Table II. Anchorage-independent growth of virus-infected fibroblast cell lines

Virus	c.f.u./ml[a] with	
	NIH/3T3	208F
MMCV	1.3 x 10^7	2.2 x 10^7
MoMuLV	0	0
Uninfected	0	0

[a]Expressed as colony-forming units per ml of supernatant from the MMCV-producing clone MC315. 3 x 10^5 cells were infected with dilutions of virus and seeded in 50 mm dishes in agarose-containing medium.

Biological activity of MMCV on fibroblasts

To determine the transforming properties of MMCV, NIH/3T3 cells were co-transfected with MMCV and MoMuLV helper virus DNAs and foci counted 4 weeks later. MMCV induced foci in these cells at 10- to 100-fold lower frequency than a similarly constructed murine retrovirus containing v-*src* (Table I). The MMCV-induced foci were small and compact, and appeared ~2 weeks later than foci induced by the v-*src*-containing virus or Harvey sarcoma virus containing v-*ras*^{H} (unpublished observation). Another *myc*-containing murine retroviral construct, which contains the same v-*myc*^{OK10} DNA fragment as MMCV but in the opposite orientation in the pMV-4 vector, failed to induce foci of transformed cells; presumably it cannot express v-*myc* due to the lack of a promoter in this transcriptional orientation. The MMCV-induced foci were isolated, recloned in agarose, and tested for virus production. Virus from one highly productive clone (MC315) was used in all subsequent experiments.

The transforming capacity of the MMCV was determined by infecting mouse NIH/3T3 cells or rat 208F fibroblasts with dilutions of virus. Infected cells were seeded into agarose and colonies counted 10 days later. Table II shows that MMCV is equally and highly efficient in inducing anchorage-independent growth in both of these cell lines. Most of the colonies were, however, considerably smaller than those induced by Harvey sarcoma virus or by the v-*src*-encoding murine retrovirus (data not shown). In addition, many cell clones had a flat morphology when grown in liquid culture. This may account for our difficulties in trying to titrate accurately the virus in standard focus assays. Such titrations have yielded focus-forming titers 3–4 orders of magnitude lower than expected from the colony-forming titers. Similar results have been obtained with virus from producer clones other than MC315. MMCV also confers anchorage-

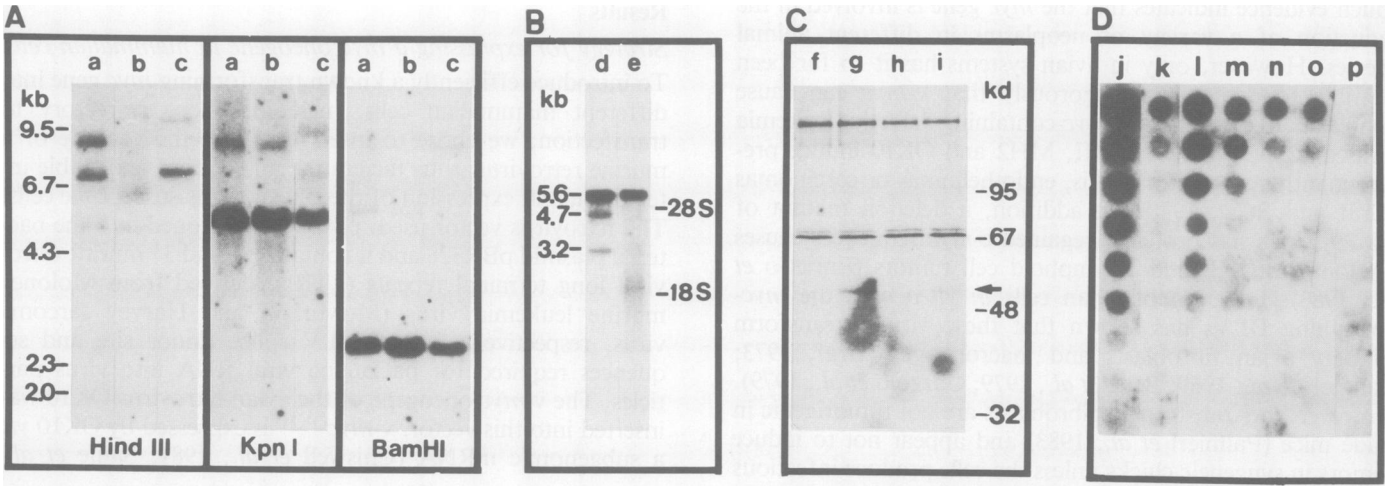

Fig. 2. Analysis of viral DNA **(A)**, RNA **(B** and **D)** and proteins **(C)** from MMCV-transformed cells. **(A)** Chromosomal DNA was cleaved with the restriction enzymes indicated and analyzed by the procedure of Southern (1975) by hybridization with a ^{32}P-labelled chicken c-*myc* probe. **Lanes a:** DNA from a MMCV-transformed NIH/3T3 clone; **b:** DNA from mass-infected NIH/3T3 cells; **c:** DNA from a transformed 208F clone. The fainter bands represent hybridization to mouse and rat c-*myc* sequences (data not shown). **(B)** Cytoplasmic polyadenylated RNA **(lane d)** and virion RNA **(lane e)** from cultures of MMCV-transformed cells were subjected to electrophoresis in denaturing agarose gels, transferred to nitrocellulose filters and hybridized with a ^{32}P-labelled chicken c-*myc* probe. **(C)** Cell extracts from ^{35}S-labelled cells were immunoprecipitated with an anti-MC29 *myc*-specific serum and analyzed by SDS-gel electrophoresis. **Lane f:** extracts from uninfected NIH/3T3 cells; **g:** MC315 cells: **h:** uninfected 208F cells: **i:** MMCV-transformed 208F cells. The arrow indicates the position of the 57-K *myc* protein. Pre-immune sera gave similar results as in **lanes f** and **h. (D)** Dot blot analysis of v-*myc*-specific RNA. Polyadenylated RNA (0.5 μg, measured spectrophotometrically) was serially diluted 2-fold and immobilized on a nitrocellulose filter. Hybridization was done with a ^{32}P-labeled *myc* probe. **lane j:** RNA from chicken fibroblasts mass-infected with MC29; **lane k:** 208F cells mass-infected with MMCV; **lane l:** clone FL5A; **lane m:** FS4C; **lane n:** FS1A; **lane o:** 311-13; **lane p:** normal chicken fibroblasts.

Table III. Transformation parameters in MMCV-transformed fibroblasts

Cell clone		Transformed morphology	Growth in agarose	Focus formation (No. of foci/100 cells seeded)	Absence of actin cables	Absence of fibronectin network	Hexose transport[a]	Level of v-*myc* expression[d]
NIH/3T3:	uninfected	−	−	N.T.	−	−	N.T.	N.T.
	MC315	++	++	N.T.	+	−	N.T.	N.T.
	TL 1	++	++	N.T.	+	−	N.T.	N.T.
	Cl 3	+	N.T.	N.T.	+	−	N.T.	N.T.
208F:	uninfected	−	−	0	−	−	1	N.T.
	FL4A[b]	++	++	30	+	+	1.5	16
	FS4C	+	++	1	+	−	1.8	4
	FS1A[b]	+	++	1	N.T.	N.T.	1.1	1.5
	F311-13	(+)[c]	+	0	−	−	1.2	1
FBR-MSV transformed 208F cells		N.T.	N.T.	N.T.	N.T.	N.T.	3.5	N.T.

++ strongly positive; + intermediate; − negative; N.T. not tested
[a]Relative to uninfected control cells.
[b]MMCV producing clones.
[c]The transformed morphology was only detectable at high cell density.
[d]The data show the relative levels of v-*myc* expression based on a RNA dot blot analysis. The expression levels were estimated from original autoradiograms, since photographic prints (such as in Figure 2D) fail to reproduce accurately.

independent growth on Rat-1 and BALB/c3T3 cells (data not shown).

Stability of the MMCV genome

To ascertain that the structure of the MMCV genome was stable upon replication, we analyzed the viral DNA, RNA and protein in transformed cells. Purified chromosomal DNA was cleaved with *Hind*III (which cleaves the proviral MMCV DNA once), *Kpn*I (cleaves in both viral LTRs) or *Bam*HI (excises the avian retroviral *myc* fragment in MMCV DNA, see Figure 1), and was analyzed by hybridization with an avian *myc* probe. Figure 2A shows the results obtained with DNA from a MMCV-transformed NIH/3T3 clone, from mass-infected NIH/3T3 cells, and from a transformed Rat-1 clone. In the *Hind*III-digested DNA the probe reacted with only one half of the proviral DNA, indicating that at least 1 − 2 copies of MMCV proviral DNA can be integrated in the host genome (lanes a and c), apparently at random positions in the mass-infected NIH/3T3 cells (lane b). The data with the *Kpn*I-digested DNA shows that the cells contained full length 5.6-kb proviral DNAs, while the *Bam*HI digests indicate that the 2.5-kb *myc*-containing fragment derived from OK10 is intact. The fainter bands present in all the digested DNAs represent hybridization to mouse and rat c-*myc* sequences, respectively (data not shown). Of ~20 MMCV-transformed clones analyzed, only one contained an aberrant (in addition to an intact) proviral genome (data not shown). We therefore conclude that the hybrid viral genome does not frequently undergo gross alterations during replication.

To study RNA expression, polyadenylated RNA was purified from the cytoplasm of a MMCV-transformed 208F clone or from virus particles released by the MC315 cells. The RNA was separated in denaturing agarose gels, transferred to filters and then hybridized with a *myc* probe. Figure 2B, lane e, shows that the virion RNA had the expected genomic length of 5.6 kb; lane d shows that genomic length viral RNA was detected in the cytoplasm together with a 4.7- and a 3.2-kb RNA. The same pattern of three intracellular viral RNAs has been found in all the 13 other clones analyzed (data not shown). The 3.2-kb RNA has a mol. wt. expected for a subgenomic mRNA having an MoMuLV RNA leader sequence spliced to the same acceptor site used for generating the OK10-encoded subgenomic *myc* mRNA. The structure of the 4.7-kb RNA cannot be explained at present.

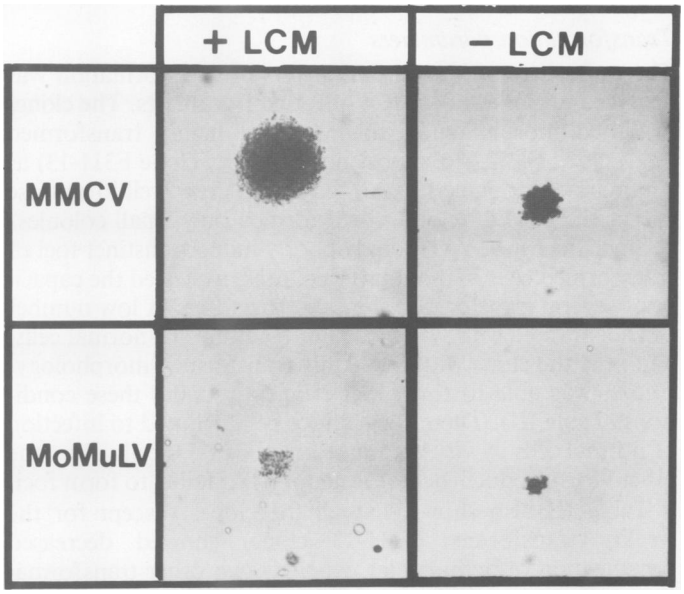

Fig. 3. Colonies of primary mouse embryo cells grown in agarose media with or without L-cell conditioned medium (LCM). Uninfected cells gave colonies similar to those in MoMuLV-infected cultures.

To show that MMCV expresses the avian v-*myc* protein, MC315 cells and an MMCV-transformed 208F clone were metabolically labelled with [^{35}S]methionine and the v-*myc* protein was immunoprecipitated with an antiserum reactive with the *myc* domain of the MC29-encoded *gag-myc* hybrid protein (M. Hayman, unpublished). Figure 2C, lanes g and i, show that a 57-K *myc* protein is present in both cell clones. In our hands, this protein is of the same size as that found in OK10-transformed chicken fibroblasts (unpublished results).

To estimate the level of *myc* expression in the MMCV-infected cells we compared the amounts of *myc* RNA in chick fibroblasts and 208F cells mass-infected with MC29 and MMCV, respectively. Figure 2D, lanes j and k, demonstrates that the MC29-transformed avian cells express 30-fold more total v-*myc* RNA than the MMCV-transformed mouse cells; when the proportion of the 3.2-kb mRNA to genomic RNA

Table IV. Transformation of mouse embryo cells by MMCV

Virus	Presence of LCM	c.f.u.[a] x 10^{-4}	Subsequent proliferative ability of colonies in liquid
MMCV	+	4.9	+
MMCV	−	0.1	−
MoMuLV	+	0.4	−
MoMuLV	−	0	

[a]Colony forming units per ml of virus inoculum. 10^5 cells were infected with dilutions of virus and seeded into agarose. Colonies were counted 10 days later.

in the latter cells is taken into account, the difference increases to ~100-fold. C-*myc* RNA in normal cells was not detectable with the hybridization conditions used (lane p).

The level of *myc* RNA varied up to 15-fold between individual MMCV-transformed 208F clones: a highly transformed clone expressed half as much total v-*myc* RNA (lane 1) as the MC29-transformed avian cells, whereas intermediately (lanes m and n) and weakly (lane d) transformed clones had proportionally less v-*myc* RNA.

Transformation parameters

The expression of several parameters of transformation was analyzed in clones of MMCV-infected fibroblasts. The clones exhibited morphologies ranging from highly transformed (e.g., clone FL5A) to almost normal (e.g., clone F311-13) as summarized in Table III. All the clones grew well in agarose except the F311-13 clone which formed only small colonies.

Since the MMCV virus only poorly induced distinct foci of transformed cells in monolayer cultures, we tested the capacity of several transformed clones to form foci. A low number of transformed cells was seeded into cultures of normal cells, and only the clone with the highly transformed morphology, FL5A, was able to form foci efficiently under these conditions (Table III). These foci cannot be attributed to infection of normal cells by virus produced by the FL5A cells, since the other virus-producing clone tested, FS1A, failed to form foci.

Table III also shows that all the clones (except for the weakly transformed F311-13 clone) showed decreased organization of actin cables, whereas two other transformation parameters tested were either only slightly altered (hexose transport) or not altered at all (fibronectin network). The FBR-MSV transformed 208F cells served as a positive control for elevated hexose transport. Comparison of the levels of v-*myc* expression (Table III) with the phenotypes of the clones suggests that the dose of *myc* is proportional to the degree of transformation.

Transformation of primary cells

The avian *myc*-encoding retroviruses readily transform primary chick embryo fibroblasts and macrophages (Graf, 1973). Primary mouse embryo cell cultures (which contain several cell types in addition to fibroblasts) were therefore infected with MMCV and then grown either in liquid medium or in agarose to monitor focus and colony formation, respectively. Conditioned medium from mouse L-cells (LCM) was included in the agarose media, since mouse macrophages depend for proliferation on a growth factor, CSF-1, produced by fibroblasts (Virolainen and Defendi, 1976; Stanley and Guilbert, 1980). Transformed cells were obtained in both types of media: the liquid cultures contained prominent foci of macrophage-like cells after 10 days, while the agarose cultures exhibited large, compact colonies that were not seen in uninfected cultures, in cultures infected with only MoMuLV or in MMCV-infected cultures lacking LCM (Figure 3). Table IV summarizes the frequencies of colony formation under the various conditions. Only colonies isolated from the MMCV-infected agarose cultures containing LCM could be further expanded in liquid medium; this also required the addition of LCM.

To establish that the cell clones were transformed by MMCV, medium was harvested from three clones and tested for the presence of transforming virus in a colony assay on 208F cells. All clones produced MMCV, thus confirming that the induction of macrophage-like colonies was dependent on infection by MMCV.

To verify that these cells were macrophages, we tested them for expression of several macrophage-specific markers. All 20 clones tested contained ~95% phagocytotic cells while cultures of uninfected mouse embryo cells contained <5%. In addition, all 10 clones tested were strongly stained in indirect immunofluorescence assays by a mouse monoclonal antibody which recognizes a cell surface antigen, mac-1 (the complement C3B receptor: Springer *et al.*, 1979), which is specific for granulocytes and macrophages. Two of the clones were also positive in immunofluorescence assays with another monoclonal antibody directed against the mac-2 antigen, which is specific for certain subpopulations of macrophages (Ho *et al.*, 1982).

None of ~100 colonies isolated from agarose cultures of MMCV-infected mouse embryo cells yielded growing cells other than macrophages; morphological transformation of fibroblast-like cells was observed in these cultures but the transformed macrophages generally overgrew the fibroblasts after several passages. To investigate further the capacity of MMCV to transform primary cells other than macrophages, cultures of limb-bud cells (which primarily contain mesenchymal cells; see Hinchliffe and Johnson, 1980) were infected with MMCV at multiplicities of infection between 0.1 and 10. Figure 4A shows fibroblast-like cells that are morphologically fully transformed two passages after infection with MMCV. When the freshly infected cells were seeded into semi-solid media, 1.8% of the cells had the capacity to grow into colonies, while 0.01% of the MoMuLV-infected cells formed colonies (Figure 4A and Table V). Large colonies of MMCV-infected cells were isolated, but have so far only yielded cultures of $<10^4$ cells before growth ceased.

To show that the transformed cells grown in liquid culture were fibroblastic, polyadenylated RNA was purified from the cultures at passage eight and tested for collagen mRNAs by Northern analysis. The MMCV-transformed cells contained collagen type I $\alpha 1$ and $\alpha 2$ mRNAs in amounts equal to normal primary cells (data not shown). Furthermore, we failed to detect any phagocytic cells in the cultures.

The normal limb-bud cells could be grown in liquid culture for 9–12 passages (10–14 generations) before the cells completely ceased growing (Figure 4B) and senesced. At this passage number most of the MMCV-infected cells also displayed a decreased growth rate and a flat morphology. However, foci of cells with high proliferative capacity appeared in these cultures or in cultures seeded at low cell density at a frequency of ~0.1% (Figure 4B). Such foci were isolated and the cells tested for proliferative ability in liquid and semi-solid media. All 10 clones tested could be expanded to cultures consisting of $>10^8$ cells (at which point the experiment was terminated), and six out of seven clones tested grew in agarose with cloning efficiencies of 10–30%. When pick-

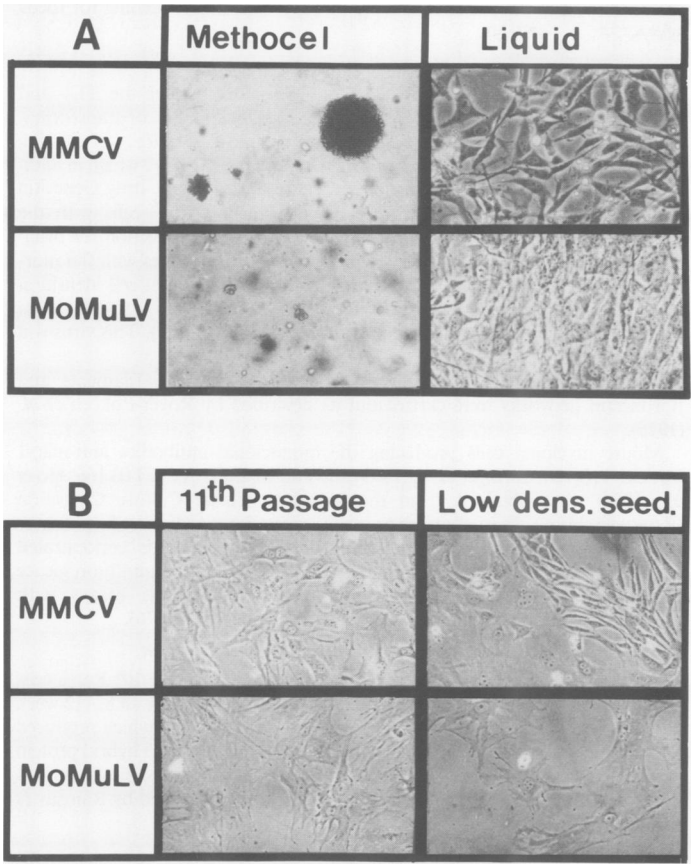

Fig. 4. Transformation of limb-bud cells by MMCV. Limb-bud cells were infected with MMCV or MoMuLV at the sixth passage after preparation and then seeded into Methocel or passaged in liquid culture. **Panel A:** the cells in liquid culture were photographed one passage after virus infection. **Panel B:** the cultures were split 1:2 when confluent and subcultured until the MoMuLV-infected cells senesced (11th passage). Cells were also seeded at low density (10^3 cells/cm^2) at the 8th passage to allow selection of cells with increased cloning and proliferative capacities.

Table V. Anchorage-independent growth of limb-bud cells transformed by MMCV

Virus	m.o.i.[a]	Number of colonies counted[b]	Percent colony forming cells
MMCV	2	5400	1.8
	0.2	1800	0.6
MoMuLV	2	30	0.01

[a]Multiplicity of infection with MMCV was determined by a colony assay using 208F cells as indicators. The m.o.i. used with MoMuLV was estimated by comparing the reverse transcriptase activity of the MoMuLV stock with that found in a stock of MMCV with a known c.f.u. titer.
[b]3 x 10^5 cells were infected with MMCV and seeded into agarose in 50 mm dishes.

ed, a majority of the colonies grew into mass cultures, which suggests that the cell clones had been established as cell lines. Collagen mRNA was detectable in three out of three clones isolated from foci, suggesting that these clones are of fibroblast origin.

Discussion

Transformation of fibroblasts from established cell lines

There may be several reasons why we have succeeded in demonstrating *myc*-induced transformation of rodent fibroblasts.

(i) We used v-*myc*^{OK10} instead of a chicken or mammalian c-*myc* gene, since v-*myc*^{OK10} transforms avian cells of different lineages *in vitro* (Beug *et al.*, 1979). Also, avian retrovirus constructs containing v-*myc*^{OK10} induce more distinct foci and larger colonies of transformed avian fibroblasts than MC29 constructs with identical LTRs (L. Frykberg and B.Vennström, unpublished observations).

(ii) The capacity of MMCV to induce focus formation is low in comparison with its ability to induce anchorage-independent growth. We therefore chose the latter, more stringent, parameter as the criterion for transformation.

(iii) With the retrovirus-mediated *myc*-expression we achieved efficient integration and expression of *myc* in a large proportion of the cells by allowing spread of virus prior to testing for focus induction. It is noteworthy that clones of transformed cells obtained by transfection with MMCV DNA, in the absence of MoMuLV helper virus DNA, have a morphology not readily distinguishable from that of normal cells and that they grow more poorly in agarose than do transformed clones obtained after infection with MMCV. This difference in transformation capacity may be due to a lower v-*myc* expression in transfected cells than in virus-infected cells, as described previously (Sherry Hwang and Gilboa, 1984).

The low capacity of MMCV to induce foci may be due to relatively low levels of v-*myc* expression in most of the infected cells, since the clones of transformed 208F cells that failed to form foci had several-fold lower levels of v-*myc* RNA than the focus-inducing clone tested (Figure 2D). The observation that the transformation phenotype is roughly proportional to the level of *myc* expression indicates that the amplified c-*myc* genes in HL60 chromosomal DNA failed to transform NIH/3T3 cells (Little *et al.*, 1983) due to low expression in the recipient cells.

Transformation of primary hematopoietic cells

The MMCV-transformed hemopoietic cells express three markers which are characteristic of macrophages: requirement of LCM for growth both in semi-solid and in liquid media, ability to phagocytose and expression of a macrophage-specific cell-surface antigen. Criteria for transformation other than anchorage-independent growth must be applied to the MMCV-transformed macrophages, since normal macrophages can also grow in an anchorage-independent manner. Some of the properties of the MMCV-transformed macrophages which distinguished them from normal cells are that they grew better than normal macrophages both in agarose (Figure 3) and in liquid and that they appeared to have an increased proliferative capacity *in vitro*.

MMCV-transformed bone marrow-derived macrophages were also shown to have properties similar to the transformed macrophages from mouse embryo cell cultures (data not shown). The ability to generate large numbers of clonally derived macrophages by infection with MMCV may facilitate studies on the diverse functions of macrophages.

Transformation of primary fibroblastic cells

Several lines of evidence indicate that the MMCV-transformed limb-bud cells are fibroblastic: mesenchymal limb-bud cells differentiate mainly into fibroblastic cells when grown at low cell densities in monolayers (Osdoby and Caplan, 1979; Hinchliffe and Johnson, 1980); the cells express collagen type I α1 and α2 mRNAs, which identified

them as connective tissue cells (Bornstein and Sage, 1980); they have a fibroblastic morphology; and finally, no phagocytic cells were detected in the cultures. Only a small fraction of the MMCV-infected cells grew as colonies, despite the high multiplicities of infection and the apparent complete morphological transformation shortly after infection. The reason(s) for this is unclear, but it is possible that only a low proportion of the cells had a lifespan long enough to permit colony formation. This explanation is supported by the fact that the MMCV-transformed cells had a high cloning efficiency in agarose media after establishment into lines.

Our notion that *in vitro* transformation of fibroblastic cells by MMCV can occur independently of 'immortalization' is consistent with the observation that primary chicken fibroblasts transformed *in vitro* by MC29 fail to yield cell lines (unpublished observations). Recent data (Land *et al.*, 1983 and H. Land, personal communication) suggest that *myc* can enhance the generation of rat embryo cell lines without inducing transformation.

MMCV transforms mammalian cells similar to the OK10 virus in the avian system

Our data show that cultured murine cells transformed by a highly active murine retrovirus encoding an avian v-*myc* oncogene have properties similar to those found in *myc*-transformed avian cells: morphological transformation and colony formation with primary fibroblasts; similar patterns of fibroblast transformation parameters (Royer-Pokora *et al.*, 1978; Palmieri *et al.*, 1982); and transformation of macrophages to increased proliferative capacity. In addition, both mouse and chicken macrophages transformed by *myc* require an exogeneous myeloid growth factor for proliferation (Figure 3 and A. Leutz, H. Beug and T. Graf, in preparation). Our data on MMCV concern only its *in vitro* transformation capacities. Additional experiments are being carried out to determine both the tumorigenicity of the *in vitro* transformed cells and the pathogenicity of the MMCV.

Three recent publications reported that proviruses found in feline lymphosarcomas have incorporated the feline *myc* gene into truncated forms of feline leukemia virus, and that these *myc*-containing viruses can infect normal cells (Levy *et al.*, 1984; Mullins *et al.*, 1984; Neil *et al.*, 1984). However, no direct evidence that these viruses have an associated transforming activity has yet been presented.

Materials and methods

Cells and viruses

NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Rat 208F fibroblasts and primary mouse cells were grown in DMEM containing 10% fetal calf serum (FCS) and 10 mM Hepes pH 7.3. The medium used for transformed macrophages was supplemented with 20% L-cell conditioned medium. Macrophages grown in liquid medium received fresh medium daily, to prevent depletion of growth factor(s). Conditioned medium was harvested from L-cells as described by Stanley and Guilbert (1980). Mouse embryo cells were prepared from 19-day old embryos of Swiss mice (Pollack *et al.*, 1974). Limb-bud cells were prepared from 12-day old embryos of NIH mice (Caplan *et al.*, 1968; Caplan, 1970).

Colony assays were done in DMEM containing 10% FCS and 0.25% agarose (Calbiochem, type A) or 1% Methocel (Fluka, novo p.s.). the serum was pre-tested for support of growth of MMCV-transformed cells in semi-solid media. Virus-infected limb-bud cells were tested for colony formation in Methocel or agarose-containing medium at $3 \times 10^4 - 10^5$ cells per ml medium. All other types of cells were tested in agarose media at a cell density not higher than $2-5 \times 10^4$ cells per ml medium in order to avoid suppression of colony formation. Reverse transcriptase assays were done as described by Goff *et al.* (1982). Transfections were carried out as described by Graham and van der Eb (1973) as modified by Stowe and Wilkie (1976). Cells transfected with MMCV and MoMuLV DNA (clone pZAP, Schoemaker *et al.*, 1981) were passaged twice in the presence of 2 μg/ml polybrene before seeding for focus assays.

Transformation parameters and cell markers

All clones of transformed NIH/3T3 or 208F cells, except the TL1 and F311-13 clones, were cloned twice in agarose before analysis of transformation parameters. All parameters were determined at least twice.

The F311-13 clone is transformed by a derivative of MMCV, which in addition to myc^{OK10} encodes a gene that confers resistance to the drug Genecitin (G418); the cell clone was obtained by transfecting 208F cells with the retrovirus DNA (without helper virus DNA) followed by selection for drug-resistant clones. This procedure gives predominantly cell clones with flat morphologies distinguishable from normal cells only at high cell densities, although virus rescued by superinfection with MoMuLV has transformation properties similar to those of MMCV (unpublished operation). This virus will be described in detail elsewhere.

Immunofluorescence studies for the detection of actin filaments and fibronectin networks were carried out as described by Royer-Pokora *et al.* (1978).

Mouse myeloma cells producing the monoclonal antibodies anti-mac-1 (ATCC TIB 128, Springer *et al.*, 1979) and anti-mac-2 (ATCC TIB 166, Ho *et al.*, 1982) were obtained from the American Tissue Culture Collection (Rockville, USA). The cells were grown in serum-free medium (Murakami *et al.*, 1982) and the antibody-containing culture supernatants concentrated 10-fold by Amicon-filtration prior to use in cell surface immunofluorescence studies (Beug *et al.*, 1979). Non-immune phagocytosis assays with macrophages were performed as described by Graf *et al.* (1976).

Preparation of myc-specific antisera and immunoprecipitation

Newborn rats (Fisher and Wistar, F) were injected with 1×10^4 Rat-1 cells transformed by MC29 (Quade *et al.*, 1983). Tumors arose over an 8 – 12 week period of time and several tumor-bearing rats were identified which produced antisera recognizing *myc*-specific determinants on the *gag-myb* hybrid protein of MC29 (Enrietto and Hayman, in preparation). Immunoprecipitation of extracts from [^{35}S]methionine-labeled cells was done as described by Ramsay *et al.* (1982).

Analysis of DNA and RNA

Nucleic acids were purified and analyzed using techniques described by Maniatis *et al.* (1982). As a *myc* probe we used a 2.3-kb *Sac*I/*Eco*RI fragment (subcloned in a derivative of pBR322) encoding exon 2 and exon 3 of chicken c-*myc*. The clone was provided by Dr. L. Frykberg.

cDNA clones specific for collagen type I, α1 and α2 (Lehrach *et al.*, 1978, 1979) were obtained from Dr. A.-M. Frischauf.

Construction of the MMC genome

First, a retrovirus vector (pMV-4) was constructed in the *Hind*III-*Bam* site of pBR328. Starting from the *Hind*III site, it contains a 1.9-kb *Hind*III-*Xba*I fragment encoding *env* and LTR sequences from Harvey sarcoma virus DNA (Ellis *et al.*, 1980), which were joined to a 1.7-kb *Xba*I-*Xho*I fragment of MoMuLV (Shinnick *et al.*, 1981) at the common *Xba*I site in their U3 sequences. The latter fragment encodes the MoMuLV splice donor site and packaging sequence. To the *Xho*I site was joined the 622 nucleotide *Sal*I-*Hind*III fragment of pBR322, which served as an adaptor molecule for ligation to a *Hind*III site introduced by addition of a synthetic linker onto a 1.1-kb *Bal*I-*Bam*HI fragment of HaSV. This fragment contains a HaSV fragment that encodes the 3′ LTR of the pMV-4 vector. The *Bam* site after this 3′ LTR was removed by partial cleavage with *Bam*HI, filling in of the protruding ends with the large fragment of DNA polymerase I, and blunt-end ligation. The vector encodes no *ras* sequences, contains a unique *Bam*HI site situated between the two LTRs, and DNA fragments up to a size of ~5.4 kb can be cloned into it without exceeding the 8.3-kb size of MoMuLV. The details for the construction of pMV-4 and the *src*-containing murine retrovirus are available from P. Luciw upon request.

To generate the MMCV construct, we added a *Bam*HI linker to the *Eco*RI site of a 2.4-kb *Bam*HI-*Eco*RI fragment encoding part of *pol* and the entire *myc* of proviral OK10 DNA (clone 401 described by Pfeifer *et al.*, 1983).

Acknowledgements

We thank Dr. Larry Guilbert for helpful discussions, Thomas Jenuwein for gift of limb-bud cells and Drs. Lars Frykberg and A.-M. Frischauf for gifts of clones. We also thank Ms. Solveig Anderson and Carmen Walter for technical assistance, and Ms. Birgit Blanasch for the typing. This project was initiated by B.V. while at the Department of Medical Genetics, Uppsala University, Sweden, and it was then funded by grants from the Swedish Natural Science Council and the Swedish National Board for Technical Development.

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Received on 11 September 1984; revised on 19 October 1984