# Modulation of Growth and Transformation of Murine MC3T3-E1 Cell Line by Murine Wild-type and Mutant p53 Genes

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## ABSTRACT

We studied the effects of murine wild-type and mutant p53 genes (p53-wt and p53val135) on the growth and transformation of murine osteoblastoid cell line MC3T3-E1. The mutant p53val135 enhanced focus formation of MC3T3-E1 cells by the activated H-*ras* plus LTR-*myc* gene and H-*ras* plus adenovirus 12 E1A gene more than four fold each, while p53-wt suppressed them 0.4 and 0.3 fold, respectively. The plating efficiency of hygromycin-resistant MC3T3-E1 cells after transfection of pSV2hygro were also increased by more than three fold with the cotransfection of p53val135 and the efficiency was also decreased 0.2 fold by cotransfection of p53-wt. These indicate that p53val135 enhances and p53-wt suppresses not only oncogene focus formation but also the cellular growth of the murine MC3T3-E1 cell line. Southern blot hybridization detected the transfected p53-wt sequence only in three out of ten MC3T3-E1 cell lines established from foci induced by p53-wt and oncogenes, and failed to detect the p53-wt DNA in hygromycin-resistant MC3T3-E1 cell lines transfected with pSV2hygro and p53-wt. These suggest that MC3T3-E1 cells containing p53-wt are at a disadvantage to form transformed foci or colonies, and suggests that MC3T3-E1 provides a good *in vitro* system to test the biological activity of murine wild-type and mutant p53 genes.

**Key words:** Murine p53 gene, Murine osteoblastoid cell line, MC3T3-E1, Transformation suppression

## INTRODUCTION

The p53 is a nuclear phosphoprotein, which is expressed at low levels in untransformed cells(1, 3) with a half-life of 6 to 30 min(14). In contrast, in

tumor-derived and transformed cell lines, the level of p53 is often elevated(2, 3) and the half-life is correspondingly extended (longer than 22 hours)(14). In SV40- and adenovirus 5-transformed cells, p53 is found in an oligomeric protein complex with the SV40 large T antigen or the adenovirus E1B 55 kilodalton (kd) protein(12, 22). The p53 gene was initially considered as a dominant growth-activating oncogene because it could cooperate with an activated *ras* gene (*ras*) in focus formation of rat embryonal fibroblasts (REF)(4, 9). However, the p53 gene with this transforming potential turned out to have a point mutation in its coding region(8), and recent studies have indicated that murine wild-type p53 (p53-wt) can suppress focus formation by transforming genes in REF and a Rat-1 cell line(5, 6, 7). On the other hand, murine p53-wt cDNA is ineffective on the plating efficiency of REF(7) and mutant p53 cDNA does not always enhance the growth of rat cells(5).

In order to further investigate and to establish an *in vitro* cell system for testing the biological function of the p53 gene, we studied the effect of murine wild-type and mutant p53 genes on the oncogene focus formation and plating efficiency of murine osteoblastoid cell line MC3T3-E1. The MC3T3-E1 cell line was established from the calvaria of newborn C57BL/6 mice(11) and it retains some of the phenotypes specific for differentiated osteoblasts. It shows high alkaline phosphatase activity in the confluent state and becomes mineralized *in vitro*(11, 17). Furthermore, similar to primary rodent cells, MC3T3-E1 is hardly transformed by *ras* alone, but they are transformed by cotransfection of *ras* and the LTR-*myc* gene (*myc*) or by *ras* and the adenovirus 12 (Ad12) E1A gene (15). By using MC3T3-E1 cells, we show here that p53-wt suppresses and mutant p53 increases both the oncogene focus formation and the plating efficiency of the cell line.

## MATERIALS AND METHODS

*Cell culture and DNA transfection*

The MC3T3-E1 cells were cultured in Dulbecco modified Eagle medium (DME) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Bio-product, MD, USA), 30 $\mu$g/m*l* of penicillin G potassium (Banyu, Tokyo, Japan) and 40 $\mu$g/m*l* of streptomycin sulfate (Meiji Seika, Tokyo, Japan). For the focus-forming assay, cells were seeded at a density of $4\times10^5$ cells per 60 mm dish, cultured for 16 to 24 hours and inoculated with oncogene plasmids (each of 2.0 $\mu$g) with or without the p53 plasmid (10 $\mu$g) by a modification of the calcium phosphate precipitation procedure(18). Three weeks after transfection, the cells were fixed by methanol and stained by Giemsa, and transformed foci were scored. Transformed cells were isolated from some of the dishes and established as

cell lines. For the assay of plating efficiency, MC3T3-E1 cells were seeded at $10^6$ cells per 100 mm dish, transfected with 6 $\mu$g of pSV2hygro and 30 $\mu$g of the p53 plasmids or 30 $\mu$g of herring testis DNA (Sigma, MO, USA) as a carrier, trypsinized and split into five dishes. Cells were then cultured in a media containing 200 $\mu$g/m*l* of hygromycin B (CALBIOCHEM, CA, USA). After ten days, hygromycin B resistant colonies were fixed with methanol and stained by Giemsa, so the colonies could be scored. Hygromycin B-resistant MC3T3-E1 cell lines transfected with pSV2hygro and p53-wt or p53val135 were also established for further analysis.

*Plasmids*

The pEJ 6.6 is a plasmid carrying the Ha-*ras* oncogene from the EJ/T24 human bladder carcinoma cell line(16). The pMoE1A is a plasmid carrying the Ad12 E1A *Hae*III-*Hae*III [1.3-5.0 map units (m. u.) of the viral genome] fragment placed downstream of the long terminal repeat (LTR) of the Moloney murine sarcoma virus (MoMSV)(19). The p12AccH is a plasmid carrying the Ad12 *Acc*IH [0-4.6 m. u.] fragment which contains the complete Ad12 E1A region(19). The pMomyc contains the second and the third exon of the mouse c-*myc* gene within the 6.0 kilobase (kb) *Bam*H1 fragment which is linked to the upstream LTR of MoMSV(19). The LTRp53cL is a plasmid carrying the mouse p53-wt cDNA (1.3 kb) placed downstream of the LTR of the Harvey murine sarcoma virus(6, 7) and it was used as the p53-wt. The LTRp53cL-val encodes a mutant p53 protein with a substitution from alanine to valine at position 135 of the LTRp53cL(6, 7) and it was used as the mutant p53 gene (p53val135). The point mutation in the LTRp53cL-val was confirmed by DNA sequencing (data not shown). The p11-4 is a plasmid carrying the mouse p53- wt cDNA driven by the SV40 promoter-enhancer(6). The pSV2hygro is a plasmid carrying a hygromycin resistant gene(10).

*Southern blot analysis*

Large-molecular-weight cellular DNA was purified from cultured cells by proteinase K digestion, phenol extraction and ethanol precipitation according to the standard method(20). Cellular DNA was digested by *Kpn*1 and *Bam*H1 (Takara Shuzo, Kyoto, Japan) at 37°C for 4 hours and fractionated by 1% agarose gel electrophoresis(20). DNA in the agarose gel was denatured in 0.4 N NaOH and 0.6 M NaCl for 40 min and transferred to a GeneScreen Plus membrane (NEN, MA, USA) according to Southern blotting(20, 21). DNA on the filter was then hybridized to a $^{32}$P-labeled p53 probe at 65°C for 18 hours in a solution containing 5× SSC (1× ; 0.15 M NaCl and 0.015 M sodium citrate), 5×

Denhardt's solution (1× ; 0.02% bovine serum albumin, 0.02% ficoll and 0.02% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate (SDS), 100 $\mu$g/m*l* of denatured herring testis DNA and 5 ng/m*l* of the labeled p53 probe. The p53 probe was prepared by labeling the 1.8 kb *Bam*H1 fragment of p53 cDNA in the p11-4 using a Random Primed DNA Labeling Kit (Boehringer Mannheim, W. Germany) with specific activity of 2 to $5\times10^8$ cpm/$\mu$g. After hybridization, the filter was washed in 2× SSC and 0.1% SDS at room temperature for 30 min, in the same buffer at 60°C for 30 min, and then in 0.1× SSC and 0.1% SDS at 55°C for 30 min and exposed to a Fuji RX X-ray film with intensifying screens.

## RESULTS

*Effect of p53-wt and p53val135 on the MC3T3-E1 cell transformation.*

To test the effect of p53-wt and p53val135 on the MC3T3-E1 cell transformation, cells were transfected with *ras* and *myc* or with *ras* and E1A in the presence or absence of p53-wt or p53val135 (Table 1). Although transformed foci were not observed in the MC3T3-E1 cell culture by transfection of *ras* alone, by *ras* and p53-wt or by *ras* and p53val135, an average of 56.5 and 23.5 transformed

**Table 1** *Effect of Murine p53-wt and p53val135 on oncogene transformation of MC3T3-E1 cells*

| DNA[1] | Number of foci[2] | | Average | Ratio |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | | |
| carrier DNA | 0 | 0 | 0 | N. A[3] |
| *ras* | 0 | 0 | 0 | N. A |
| *ras*+p53-wt | 0 | 0 | 0 | N. A |
| *ras*+p53val135 | 0 | 0 | 0 | N. A |
| *ras*+*myc* | 53 | 60 | 56.5 | 1.0 |
| *ras*+*myc*+p53-wt | 24 | 23 | 23.5 | 0.42 |
| *ras*+*myc*+p53val135 | 255 | 258 | 256.5 | 4.53 |
| *ras*+E1A | 23 | 24 | 23.5 | 1.0 |
| *ras*+E1A+p53-wt | 8 | 7 | 7.5 | 0.32 |
| *ras*+E1A+p53val135 | 103 | 107 | 105.0 | 4.47 |

[1] Input DNA was adjusted to 14 $\mu$g by herring testis (carrier) DNA.

[2] MC3T3-E1 cells were seeded at $4\times10^5$ cells per 60 mm dish, then transfected with oncogene plasmids (each 2.0 $\mu$g) with or without p53 plasmid (10 $\mu$g). The total number of foci in three dishes was scored three weeks after transfection.

[3] not applicable

foci per three dishes were induced in MC3T3-E1 cells transfected with *ras* and *myc* and with *ras* and E1A, respectively (Table 1). Additional cotransfection of p53-wt suppressed the MC3T3-E1 transformation by *ras* and *myc* (23.5 foci) and by *ras* and E1A (7.5 foci) (Table 1). On the other hand, cotransfection of p53val135 enhanced the focus formation of MC3T3-E1 transfected with *ras* and *myc* (256.5 foci) and with *ras* and E1A (105.0 foci) (Table 1). Representative results of focus formations of MC3T3-E1 with or without p53 genes are shown in Fig. 1. The p53val135 has been reported to be a temperature-sensitive (ts) mutant and it behaves as a wild-type p53 gene at low (32.5°C) temperatures(13). Similar to the experiment using REF(13), MC3T3-E1 cells transfected with *ras*, *myc* and p53val135 and cultured at 33°C produced transformed foci with an

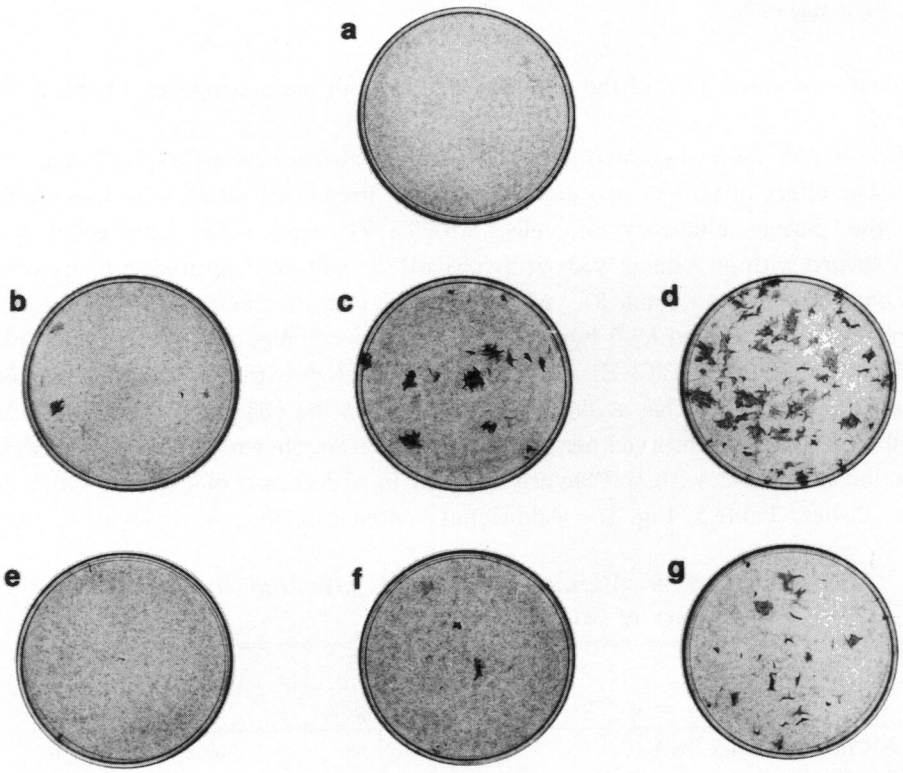


**Fig. 1** MC3T3-E1 cell cultures transfected with oncogenes in the presence or absence of p53 genes. Cells were fixed by methanol and stained by Giemsa three weeks after transfection. a, MC3T3-E1 cells transfected with carrier DNA.; b, MC3T3-E1 with *ras*, *myc* and p53-wt.; c, MC3T3-E1 with *ras* and *myc*.; d, MC3T3-E1 with *ras*, *myc* and p53val135.; e, MC3T3-E1 with *ras*, E1A and p53-wt.; f, MC3T3-E1 with *ras* and E1A .; g, MC3T3-E1 with *ras*, E1A and p53val135.

**Table 2** *Transformation of MC3T3-E1 cells by ras and myc with or without p53-wt or p53val135 at 33°C*

| DNA[1] | Number of foci[2] | | Average | Ratio |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | | |
| carrier DNA | 0 | 0 | 0 | N. A[3] |
| *ras*+*myc* | 41 | 31 | 36 | 1.0 |
| *ras*+*myc*+p53-wt | 10 | 11 | 10.5 | 0.29 |
| *ras*+*myc*+p53val135 | 18 | 17 | 17.5 | 0.49 |

[1] Input DNA was adjusted to 14 $\mu$g by herring testis (carrier) DNA.
[2] MC3T3-E1 cells were seeded at $4\times10^5$ cells per 60 mm dish, then transfected with oncogene plasmids (each 2.0 $\mu$g) with or without p53 plasmid (10 $\mu$g). The total number of foci was scored three weeks after transfection.
[3] not applicable

efficiency of about 50% of the cells transfected with only oncogenes (Table 2).

*Effect of p53-wt and p53val135 on the plating efficiency of MC3T3-E1 cells*

The effect of p53-wt and p53val135 on the growth of MC3T3-E1 was studied by the plating efficiency of cells. MC3T3-E1 cells were transfected with pSV2hygro with or without p53-wt or p53val135, split and cultured in hygromycin B-containing media (Table 3). MC3T3-E1 cells transfected with pSV2hygro and carrier DNA produced 69.0 hygromycin-resistant colonies per five dishes (Table 3, Fig. 2). When MC3T3-E1 cells were transfected with pSV2hygro and *Stu*1 cleaved p53-wt (*Stu*1 has a single cleavage site in the p53 cDNA of LTRp53cL), 70.0 colonies were observed per five dishes (data not shown). Additional cotransfection of p53-wt with pSV2hygro resulted in a decrease of colonies (15.5 per five dishes, Table 3, Fig. 2). Additional cotransfection of p53val135 with

**Table 3** *Effect of Murine p53-wt and p53val135 on the plating efficiency of MC3T3-E1 cells*

| DNA | Number of colonies[1] | | Average | Ratio |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | | |
| pSV2hygro+carrier DNA | 80 | 58 | 69.0 | 1.0 |
| pSV2hygro+p53-wt | 17 | 14 | 15.5 | 0.22 |
| pSV2hygro+p53val135 | 259 | 240 | 249.5 | 3.62 |

[1] MC3T3-E1 cells were seeded at $10^6$ cells per 100 mm dish, transfected with 6 $\mu$g of pSV2hygro and 30 $\mu$g of p53 plasmid or carrier DNA, then split into five dishes and cultured in hygromycin B (200 $\mu$g/m*l*) media. Hygromycin B-resistant colonies were scored 10 days after transfection.

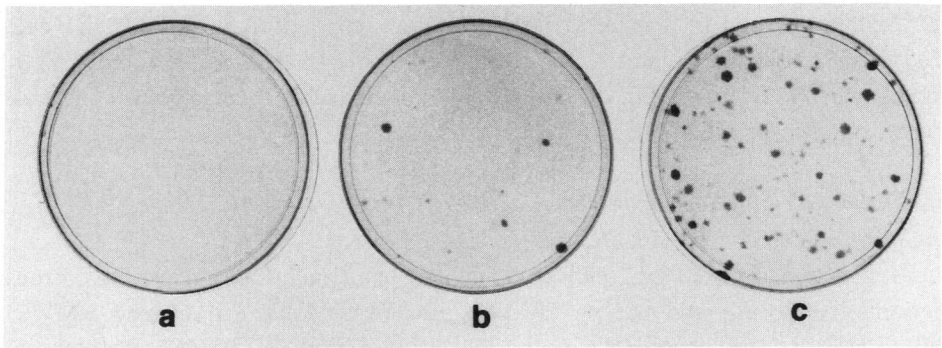


**Fig. 2** MC3T3-E1 cell colonies transfected with pSV2hygro with or without p53 genes and cultured in the hygromycin B-containing media. Cells were fixed by methanol and stained by Giemsa ten days after transfection. a, MC3T3-E1 cells transfected with pSV2hygro and p53-wt.; b, MC3T3-E1 with pSV2hygro and carrier DNA.; c, MC3T3-E1 with pSV2hygro and p53val135.

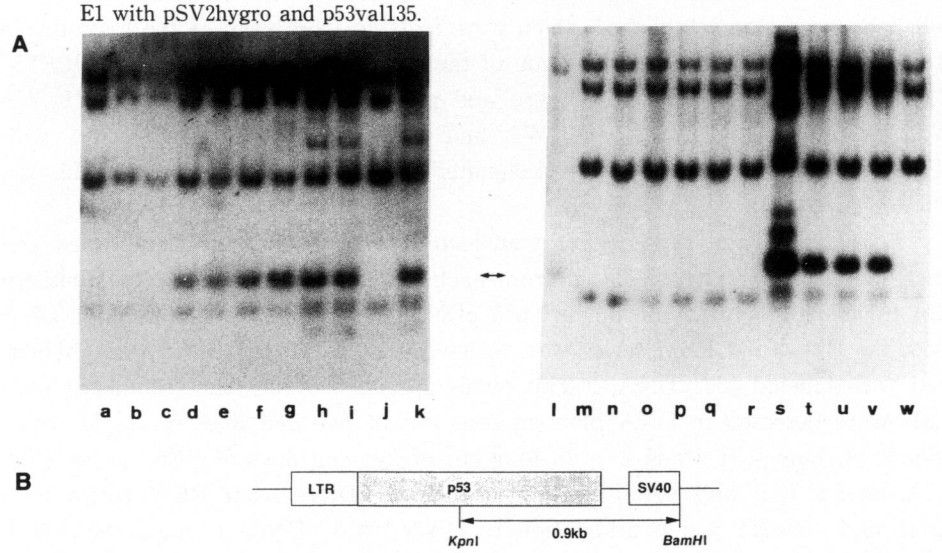


**Fig. 3** A, Southern blot hybridization of transformed cell lines. Ten $\mu$g of cellular DNA from transformed MC3T3-E1 cell lines were digested with *Kpn*1 and *Bam*H1, electrophoresed in 1.0% agarose gel and transferred to a GeneScreen Plus membrane by Southern blotting. DNA blots were hybridized to a $^{32}$P-labeled p53 probe, washed and exposed to X-ray film as described in materials and methods. An arrow indicates the 0.9 kb band derived from the transfected p53 sequence. Lane a, MC3T3-E1.; b, RMW1.; c, RMW2.; d, RMW3.; e, RMW4.; f, RMW5.; g, RMM1.; h, RMM2.; i, RMM3.; j, RMM4.; k, RMM5.; l, LTRp53cL DNA equivalent to one copy per cell and 10 $\mu$g of herring testis DNA which was cleaved with *Kpn*1 and *Bam*H1.; m, MC3T3-E1.; n, REW1.; o, REW2.; p, REW3.; q, REW4.; r, REW5.; s, REM1.; t, REM2.; u, REM3.; v, REM4.; w, REM5. B, Restricition sites of *Bam*H1 and *Kpn*1 on the LTRp53cL. Viral and cellular sequences are indicated by boxes and plasmid sequences by lines.

pSV2hygro resulted in a enhancement of colony formation of MC3T3-E1 cells (249.5 per five dishes, Table 3, Fig. 2). These indicate that the effect of p53-wt and p53val135 on the growth of MC3T3-E1 is comparable to that on the oncogene transformation of MC3T3-E1.

*Establishment and Southern blot analysis of transformed MC3T3-E1 cell lines and hygromycin B resistant MC3T3-E1 cell lines*

Transformed MC3T3-E1 cell lines isolated from each foci induced by cotransfection of *ras*, *myc* and p53-wt (RMW1, RMW2, RMW3, RMW4 and RMW5), cell lines by *ras*, *myc* and p53val135 (RMM1, RMM2, RMM3, RMM4 and RMM5), cell lines by *ras*, E1A and p53-wt (REW1, REW2, REW3, REW4 and REW5) and cell lines by *ras*, E1A and p53val135 (REM1, REM2, REM3, REM4 and REM5) were established for further analysis. Untransformed MC3T3-E1 cell lines were also established which were isolated from each of the hygromycin B resistant colonies after transfection of the pSV2hygro and p53 genes. MC3T3-E1 lines transfected with pSV2hygro and p53-wt were designated as W1, W2, W3, W4, W5, W6, W7, W8 and W9 and MC3T3-E1 lines transfected with pSV2hygro and p53val135 were designated as M1, M2, M3, M4, M5, M6, M7, M8 and M9.

To examine the presence of transfected p53 sequences in established cell lines, cellular DNA was isolated from each cell line and analyzed by Southern blot hybridization with the labeled p53 cDNA as a probe. When cellular DNA from the RMM and REM series was digested with *Kpn*1 and *Bam*H1 and hybridized with labeled p53 cDNA, 0.9 kb bands specific for the transfected p53 plasmids were detected in DNA blots of four out of five cell lines of RMM series (Fig. 3, A, lane g, h, i and k). In four out of five cell lines of REM series (Fig. 3, A, lane s, t, u and v). Digestion of cellular DNA from REW series with *Kpn*1 and *Bam*H1 and hybridization to labeled p53 cDNA detected the 0.9 kb bands in three out of five RMW series (Fig. 3, A, lane d, e and f), while a hybridized band was not detectable in the REW series (Fig. 3, A, lane n, o, p, q and r). When cellular DNA from the M cell lines was cleaved with *Kpn*1 and *Bam*H1 and hybridized with labeled p53 cDNA, 0.9 kb bands from p53val135 were observed in four out of nine cell lines (Fig. 4, lane o, q, s and t). On the other hand, DNA blots from W cell lines did not contain the 0.9 kb fragment hybridized to the p53 probe (Fig. 4, lane c, d, e, f, g, h, i, j and k). Cells containing the exogeneous p53-wt had a tendency of growth disadvantage and failed to form a transformed focus or a colony.

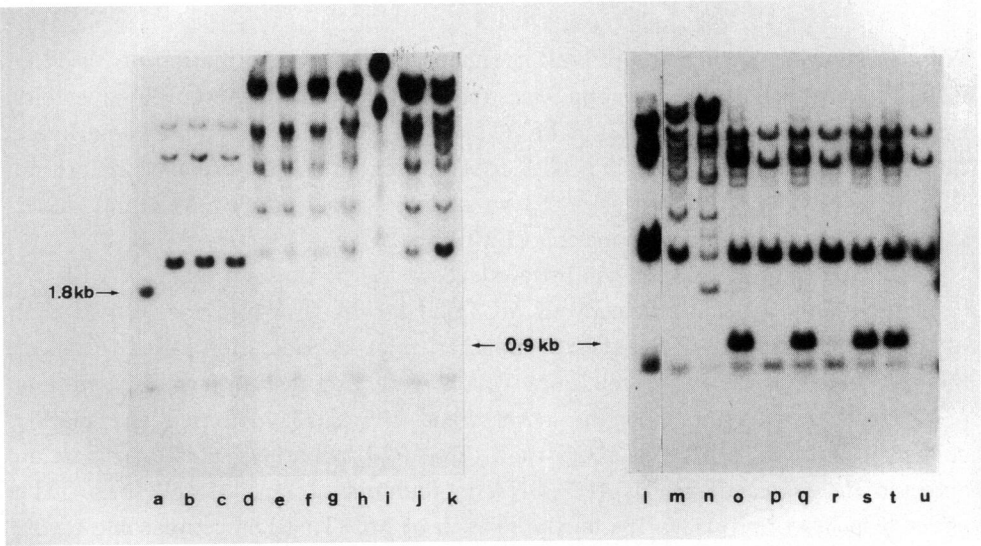


**Fig. 4** Southern blot hybridization of hygromycin B resistant MC3T3-E1 cell lines. Ten $\mu$g of cellular DNA from hygromycin B resistant MC3T3-E1 cell lines was digested with *Kpn*1 and *Bam*H1, electrophoresed in 1.0% agarose gel and transferred to a Gene-Screen Plus membrane by Southern blotting. DNA blots were hybridized to a $^{32}$P-labeled p53 probe, washed and exposed to X-ray film as described in materials and methods. Lane a, the 1.8 kb *Bam*H1 fragment of p53 cDNA in the p11-4 (one copy per cell).; b, MC3T3-E1.; c, W1.; d, W2.; e, W3.; f, W4.; g, W5.; h, W6.; i, W7.; j, W8.; k, W9.; l, M1.; m, M2.; n, M3.; o, M4.; p, M5.; q, M6.; r, M7.; s, M8.; t, M9.; u, MC3T3-E1. Refer to the cleavage map of the p53 plasmid in Fig. 3.

## DISCUSSION

Previous studies have demonstrated that murine p53-wt suppressed a oncogene transformation of REF(5, 6, 7) and a Rat-1 cell line(7). We showed that murine p53-wt also suppressed a focus formation of MC3T3-E1 cells transfected with *ras* and *myc* and with *ras* and E1A. The suppression of oncogene focus formation of MC3T3-E1 cells by p53-wt cotransfection (0.3 to 0.4 fold) was more effective than that of a Rat-1 cell line previously reported (0.75 fold)(7). Southern blot hybridization detected transfected p53-wt sequences in only three out of ten cell lines established from foci induced by p53-wt and oncogenes, and in eight out of ten cell lines isolated from foci induced by p53val135 and oncogenes which carried the transfected p53val135 sequence. None of the five REW lines isolated from foci by p53-wt, E1A and *ras* contained p53-wt DNA. These suggest that oncogene transformed MC3T3-E1 cells containing exogeneous p53-wt were at a disadvantage to form transformed foci. The murine mutant p53 gene enhances oncogene focus formation of REF and the Rat-1 cell line(5, 6, 7).

We also showed that murine p53val135 enhanced the focus formation of MC3T3-E1 cells transfected with *ras* and *myc* and with *ras* and E1A four to five fold. Enhancement of transformation of MC3T3-E1 by p53val135 was more remarkable than that of the Rat-1 cell line (1.6 fold). Similar to the previous report on REF(13), p53val135 behaved as a p53-wt at low temperatures (33°C) and rather suppressed oncogene focus formation of MC3T3-E1 cells.

Although murine p53-wt has little effect on the plating efficiency of REF(7), it decreased the plating efficiency of MC3T3-E1 cells (0.2 fold) as well as that of the Rat-1 cell line (0.4 fold)(5). Southern blot hybridization failed to detect the p53-wt sequence in W cell lines which were established from p53-wt and pSV2hygro transfection. On the other hand, p53val135 increased the plating efficiency of MC3T3-E1 cells more than three fold. Southern blot hybridization detected the transfected p53val135 DNA in four out of nine M cell lines. The effect of p53-wt and p53val135 on the growth of MC3T3-E1 is comparable to the effect on the oncogene transformation of MC3T3-E1. This is different from the result in REF or the Rat-1 cell line. The plating efficiency of REF is hardly suppressed by p53-wt(7). In the Rat-1 cell line, the plating efficiency is suppressed by p53-wt, while it is not enhanced by the murine mutant p53 gene where point mutations reside at 168 and 234 codons(5).

The MC3T3-E1 cell line possesses phenotypes characteristic of primary osteoblasts including high alkaline phosphatase activity, extracellular calcification and a resistance to *ras* gene transformation. Our present results describe a sensitive response of the MC3T3-E1 cell line to p53 gene functions, and it was suggested that the MC3T3-E1 cell line provides a good assay system for the wild-type and mutant p53 gene activity.

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