*Tumor Res.* **21**, 67–76 (1986)

# The LTR-linked Adenovirus Type 12 E1B Gene Enhances Some Transforming Gene Focus Formations in Mouse NIH3T3 Cell Culture

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## SUMMARY

The LTR of Moloney murine sarcoma virus was linked to the upstream of the adenovirus type 12 (Ad12) E1B sequence (4.47–16.24 map units [m. u.]) to construct a recombinant plasmid pMoE1B, and 3T3B1 cells expressing Ad12 E1B was established by introducing the pMoE1B into NIH3T3 cells. Cotransfection (NIH3T3 cells) and transfection (3T3B1 cells) experiments showed that pMoE1B enhances the focus-formation of the Ad12 E1A, Ad4 E1A, and mutated SV40 early genes in NIH3T3 cell culture. No focus-formation was observed by transfection of the polyomavirus large T or LTR-connected mouse c-*myc* genes in 3T3B1 cells.

**Key words:** Adenovirus type 12 E1B sequence, Transformation

## INTRODUCTION

Human adenoviruses can transform rodent cells *in vitro* (1). DNA transfection studies showed that complete cell transformation could be induced by the left-end 6.8% of the adenovirus genome, the early region 1A (E1A, 0–4.5 map units [m. u.]) and a part of the E1B (4.6–6.8 m. u.) (2). The E1A gene product is required for efficient expression of the E1B region and other early regions of the viral genome (3, 4). The E1A region induces incomplete transformation and immortalization of primary cells (5, 6). However, the E1B region (4.6–11.2 m. u.) with or without linked SV40 early promoter-enhancer cannot transform primary or established rat cells (7). In this paper, we constructed the recombinant pMoE1B, the Ad12 E1B combined with the LTR of Moloney murine sarcoma virus (MoMSV), and the 3T3B1 cell line was established by introducing the recombinant into NIH3T3 cells. The recombinant and the 3T3B1 cell line were used for cooperative transformation studies.

## MATERIALS AND METHODS

*Cell culture and DNA transfection experiments*

A mouse NIH3T3 cell line, kindly provided by Dr. Mitsuaki Kakinuma of Hokkaido University, and its transformed derivatives were cultured in Dulbecco's modified Eagles medium (DMEM) with 7% fetal bovine serum (FBS, Flow Laboratories). DNA transfections were performed essentially by the calcium phosphate technique as described(8). For the establishment of NIH3T3 lines containing the LTR-E1B sequences, NIH3T3 cells ($5\times10^5$ cells/6 cm dish, two dishes) were transfected with 1.0 $\mu$g of pSV2*neo* and 16.0 $\mu$g of *Eco*RI-cleaved pMoE1B (1 : 10 molar ratio). This was cultivated for 20 hrs, split at one tenth into 10 cm dishes and cultured in media containing the antibiotic G418(9) (400 $\mu$g/m*l*). After a two-week-culture, G418-resistant colonies were isolated and established into cell lines (total twelve lines). For the study of focus-forming activity, $2\times10^5$ cells were seeded in a 6 cm dish and cultured for 24 hrs. Then the cells were transfected with recombinant plasmid DNA (super-coiled), incubated for 18-20 hrs and fed with fresh medium. After three weeks, and changing media (DMEM with 3% FBS) every three days, cells were fixed with methanol and stained with Giemsa for counting transformed cell foci.

*Recombinant plasmids*

A recombinant plasmid pSV2*neo*, kindly provided by Dr. Maurice Green of St. Louis University, is a derivative of pSV2neo(10) and has a single *Bam*HI site at the 3′ end of the inserted transcription unit. pSV2neo and pSV2*neo* have a neomycin resistant gene combined with SV40 early promoter-enhancer region. To construct a plasmid p12AccH, the Ad12 *Acc*IH fragment (0-4.7 m. u.) was cloned into pBR322 at the site of *Eco*RI and *Acc*I (2246 nucleotide position [n. p.]) by using *Eco*RI linker at the original end of the viral DNA. A plasmid p5E1a containing the Ad5 *Xba*I-E fragment (0-4.5 m. u.) cloned between the *Eco*RI and the *Pvu*II (2067 n. p.) sites of pBR322 was described elsewhere(11). The plasmid pKA4(12) was a gift of Dr. Morikazu Shinagawa of Obihiro University of Agriculture and Veterinary Medicine. This contains the Ad4 *Bam*HI-F fragment (0-5.9 m. u.) integrated between the *Bam*HI and the *Sal*I sites of pBR322. Recombinant plasmids pMTAI and pMTAII, gifts of Dr. Nobuo Yamaguchi of University of Tokyo, have the SV40 sequences of the entire early region (0-0.712 m. u. of SV40 genome) with mutations at *Ava*II sites of 0.636 and 0.335 m. u., and with only immortalization and incomplete transformation activity, respectively(13). To construct a plasmid pMomyc, 5.5 kilobase (kb)-*Bam*HI fragment of murine c-*myc* sequence(14) containing a part of the second intron, the second and the third exon and the poly(A) signal sequences was cloned into the *Bam*HI site of pMoLTR at the

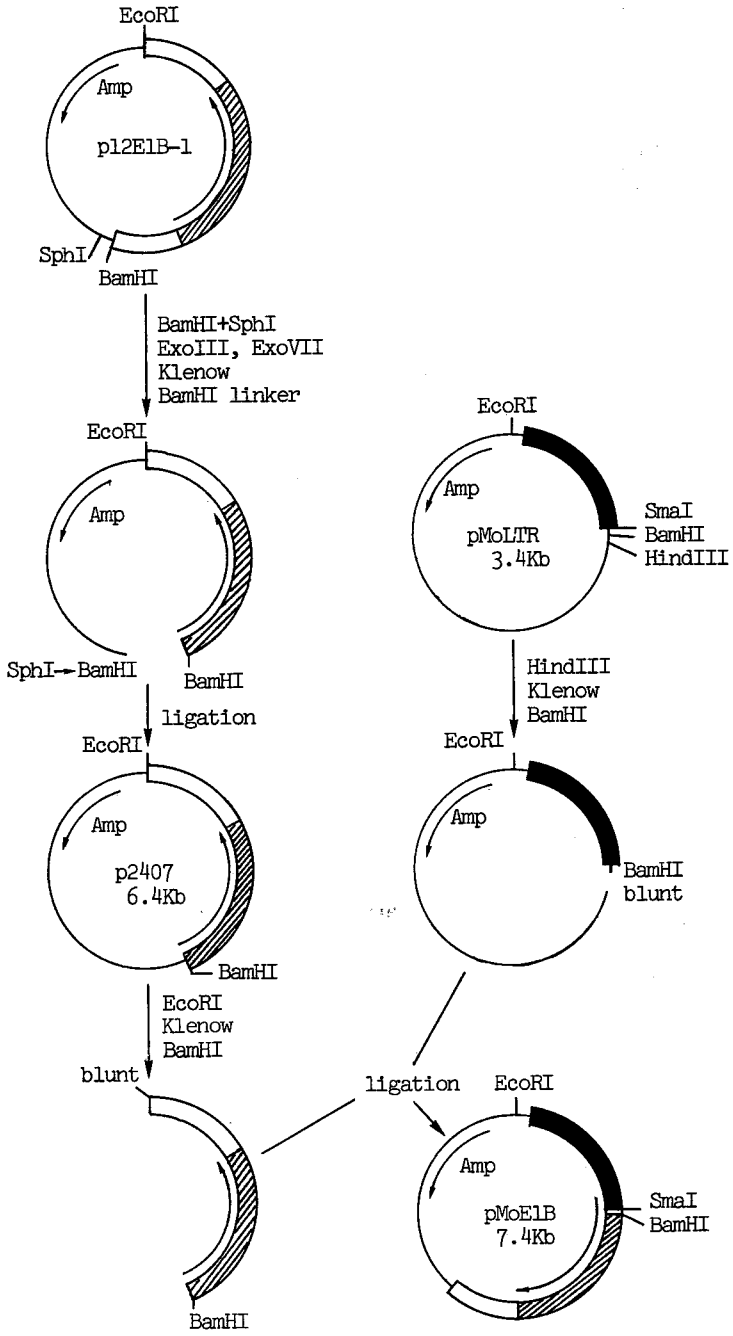


**Fig. 1** Construction of a recombinant plasmid pMoE1B containing the Ad12 E1B region (▨) with the MoMSV-LTR sequences (■).

downstream of the LTR of MoMSV. A plasmid pMoLTR was constructed on pUC8 that contains LTR sequence of MoMSV(15) from the *Eco*RI site (472 base pairs [bp] upstream from the transcriptional start site) to the *Sma*I site (28 bp downstream from the transcriptional start site) integrated between the *Eco*RI and the *Sma*I sites of pUC8.

pPyLT, a gift of Dr. Kaoru Segawa of Keio University, has the polyomavirus DNA without the intron sequence (0.78–0.86 m. u. of the polyomavirus genome) of the large-T coding gene which was linearized by *Bam*HI at 0.58 m. u. and inserted into the *Bam*HI site of pBR322. pEJ6.6(16) contains 6.6kb-*Bam*HI fragment of human c-Ha-*ras* 1 gene from the bladder carcinoma cell line EJ. A recombinant plasmid pMoE1B was constructed as follows: First, a plasmid p2407 was made from p12E1B-1 which is a recombinant containing the Ad12 *Kpn*I (588 n. p.)-*Eco*RI (5570 n. p.) fragment between the *Eco*RI and the *Bam*HI sites of pBR322 in such a way that the *Kpn*I site was converted to the *Bam*HI site after ligation. p12E1B-1 was digested with *Bam*HI and *Sph*I, treated with ExoIII, ExoVII and DNA polymerase I (Klenow fragment) to delete the E1A region and to make both ends blunt. It was then ligated with *Bam*HI linker and circularized to construct p2407. A plasmid p2407 has the Ad12 sequence from 4.47 m. u. (1532 n. p.) to 16.24 m. u. containing the E1B coding region of which the leftside terminus (1532 n. p.) was determined by DNA sequencing. The Ad12 E1B sequence cut out from p2407 by *Eco*RI and *Bam*HI digestion were recloned into the *Bam*HI site of pMoLTR as shown in Fig. 1.

*Northern blot hybridization*

Total cytoplasmic RNA was prepared from cultured cells by the method described(17). Northern blot hybridization was carried out by the method of Rave *et al.*(18) and Thomas(19) with a slight modification(20). After 50 $\mu$g of cytoplasmic RNA was electrophoresed in a 1.2% agarose gel containing 6% formaldehyde, 0.018 M $Na_2HPO_4$ and 0.002 M $NaH_2PO_4$, RNA was transferred to a nitrocellulose filter according to the Southern technique(21). RNA blots were prehybridized with 50 $\mu$g/m*l* of sonicated and denatured salmon sperm DNA in hybridization buffer described previously(20) at 45°C for 6 hrs, followed by hybridization with 50 ng of $^{32}$P-labeled viral or recombinant plasmid DNA (prepared by nick translation) at 45°C for 20 hrs. Then the filters were washed and hybridized RNA was detected by autoradiography.

*Materials*

Restriction enzymes and synthetic DNA linkers were purchased from Takara Schuzo Co. Ltd.. T4 ligase, ExoIII and ExoVII were products of Bethesda Research Laboratories. Nick translation kits and DNA polymerase I (Klenow fragment)

was from Boehringer-Manheim Corporation. Calf intestine phosphatase was obtained from Sigma and the antibiotic G418 from Gibco Laboratories.

## RESULTS

*Establishment of NIH3T3 cell lines containing the LTR-Ad12E1B sequence*

A plasmid pMoE1B containing the Ad12 E1B sequence (4.47–16.24 m. u.) with the MoMSV-LTR was constructed from p12E1B-1 and pMoLTR as shown in Fig. 1. Left-side terminus of the viral insert (1532 n. p. of the viral genome) is located at 34bp downstream from the TATA box (TATAAAG at 1498 n. p.) and at 9bp upstream from the E1B 19 kilodalton(k) initiation triplet (ATG at 1541 n. p.) in an alignment of the Ad12 E1B(22). Murine NIH3T3 cells were cotransfected with the plasmid pMoE1B and pSV2*neo* and cultured in the antibiotic-G418 containing media. Among twelve G418-resistant cell lines, at least six lines contained E1B mRNA and one of them, designated as 3T3B1 line, was found to contain abundant E1B transcripts (Fig. 2). All of the six cell lines expressing the E1B mRNA showed the fibroblastoid morphology similar to that of parental NIH3T3 cells, reached saturation in monolayers and formed no colonies in 0.3% agar suspension culture. This indicates that Ad12 E1B gene has no detectable transforming ability in NIH3T3 cells.

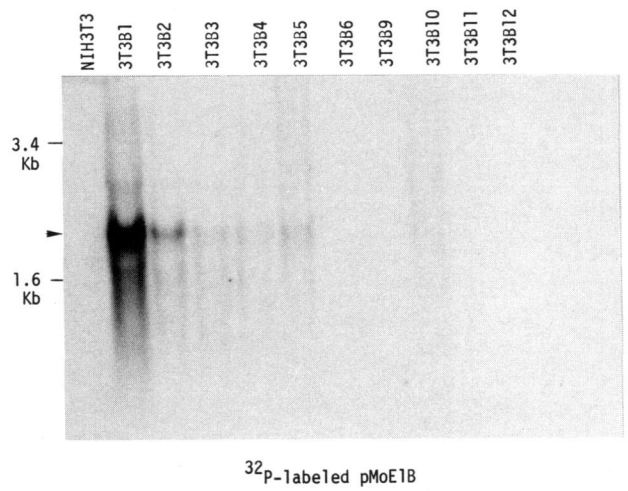


**Fig. 2** Northern blot hybridization of RNA from G418-resistant NIH3T3 lines (3T3B1-3T3B12) obtained by the cotransfection with pSV2*neo* and pMoE1B. RNA blots (50 $\mu$g of total cytoplasmic RNA per lane) were hybridized with $^{32}$P-labeled pMoE1B. The arrow head indicates the Ad12 E1B mRNA.

*Enhancement of viral transforming gene focus-formation by the pMoE1B in NIH3T3 cells*

As shown in Table 1, recombinant plasmids p12AccH, p5E1a, pMTAI, pMTAII, pPyLT and pMomyc did not induce or hardly induced transformed foci on murine NIH3T3 cell culture, and the plasmid pKA4 induced transformed foci with a rather low efficiency (3.3 foci/$\mu$g DNA). However, when cotransfected with pMoE1B, p12AccH and pKA4 induced transformed foci with efficiencies more than

**Table 1** *Focus-formation of NIH3T3 and 3T3B1 cells with recombinant plasmids.*

| | Transfected DNA | ug/dish | Number of foci[1] NIH3T3 | 3T3B1 |
|---|---|---|---|---|
| Exp. I | p12AccH | 3.0 | 0, 0 | 2, 5 |
| | pMomyc | 3.0 | 0, 0 | 0, 0 |
| | pMTAI | 3.0 | 0, (2)[2] | 0, 0 |
| | pMTAII | 3.0 | 1, 2 | 10, 15 |
| | EJras | 0.1 | >200, >200 | >200, >200 |
| | pUC19 | 3.0 | 0, 0 | 0, (1) |
| Exp. II | p5E1a | 5.0 | N. T.[3] | 1, 2 |
| | pKA4 | 5.0 | N. T. | 125, 132 |
| | pPyLT | 5.0 | 0, 0 | 0, 0 |
| | p12AccH | 5.0 | 0, 0 | 4, 6 |
| | pMTAII | 5.0 | 2, 7 | 22, 25 |
| | pMoE1B | 5.0 | 0, (1) | 0, 0 |
| | p12AccH+pMoE1B | 5.0+5.0 | 57, 74 | N. T. |
| | pMTAII+pMoE1B | 5.0+5.0 | 5, 11 | N. T. |
| | pUC19 | 5.0 | 0, 0 | 0, 0 |
| Exp. III | p12AccH | 5.0 | 0, 1 | |
| | p5E1a | 5.0 | 0, 2 | |
| | pKA4 | 5.0 | 15, 18 | |
| | pMoE1B | 5.0 | 0, 0 | |
| | p12AccH+pMoE1B | 5.0+5.0 | 126, 156 | |
| | p5E1a+pMoE1B | 5.0+5.0 | 2, 2 | |
| | pKA4+pMoE1B | 5.0+5.0 | >200, >200 | |
| | pUC19 | 5.0 | 0, 0 | |

1) $2\times10^5$ cells in a 6 cm dish were transfected with supercoiled DNA (except for EJras DNA), incubated for 20 hrs at 37°C, fed with fresh media and cultured for three weeks.

2) Numbers in parentheses are foci of spontaneously transformed cells which are easily distinguishable from their morphology. Three lines obtained in Exp. I did not contain viral or plasmid sequences.

3) N. T.: not tested

a hundred fold and the plasmid pMTAII with an efficiency of about two fold compared to those by a single gene transfection. p12AccH, pKA4 and pMTAII contain the viral seqeuences of Ad12E1A, Ad4E1A and mutated SV40 early sequences with only an incomplete transformation ability, respectively. Rat transformants by these transforming genes showed limited growth characteristics in soft-agar media(5, 13, 23). Morphology of the transformed foci induced by cotransfection (Fig. 3) were similar to those induced by the adenovirus E1A plasmid alone, or pMTAII alone respectively.

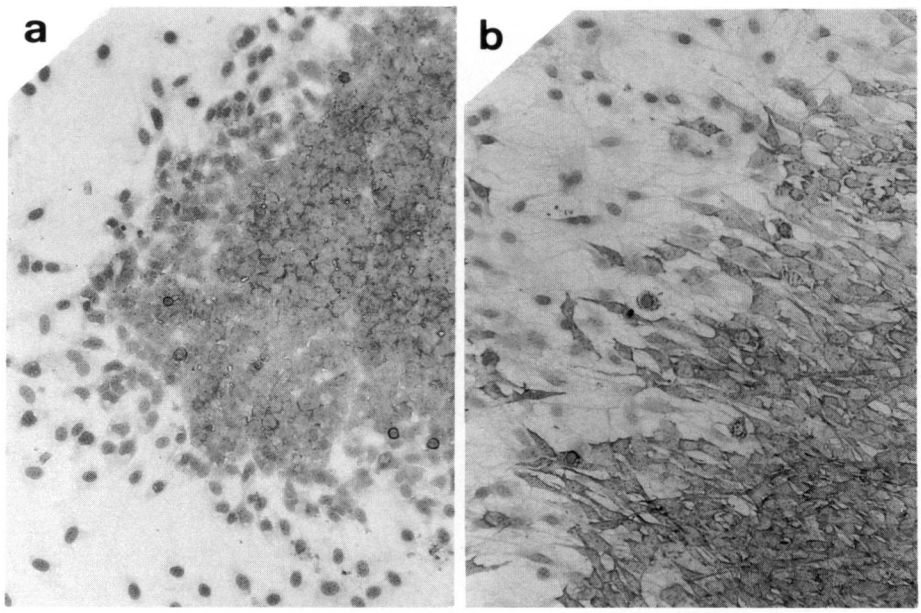


**Fig. 3** Transformed foci in NIH3T3 cells induced by cotransfection of p12AccH and pMoE1B(a) and by cotransfection of pMTAII and pMoE1B(b).

Then, cooperations of different transforming genes with the Ad12 E1B were investigated by transfection experiments of 3T3B1 cells which carried the Ad12 E1B function. Recombinant plasmids p12AccH, pKA4 and pMTAII again induced transformed foci on 3T3B1 cell culture more efficiently than on NIH3T3 culture (Table 1). On the other hand, no transformed foci were observed when 3T3B1 cells were transfected with plasmids p5Ela, pMTAI, pPyLT and pMomyc. Viral and cellular transforming genes in these plasmids can only immortalize primary rodent cells and/or transform primary cells cooperatively with a human activated *ras* gene(6, 13, 24). A 3T3B1 cell line transformed by p12AccH (designated as 3T3B1H2), by pKA4 (designated as 3T3B14A3, 3T3B14A5, 3T3B14A6 and 3TB14A7) and by pMTAII (designated as 3T3B1MTII1, 3T3B1MTII3,

3T3B1MTII4, 3T3B1MTII6 and 3T3B1MTII7) were established from transformed foci. Cytoplasmic RNA was prepared from each of the transformed cell lines and examined for the presence of the viral mRNA by Northern blot hybridization. A 3T3B1H2 cell line and four 3T3B14A cell lines contained about 1.0kb-E1A mRNA hybridized with probes of labeled Ad12 E1A and Ad4 E1A plasmid DNA, respectively (Fig. 4). In the RNA blots of 3T3B1MTII lines, RNA bands specific to SV40 sequences were detected by hybridization with the labeled pMTAII DNA (Fig. 4). As a result, 3T3B1 cells transformed efficiently by transfection of recombinant plasmids contained viral transcripts from viral DNA inserts.

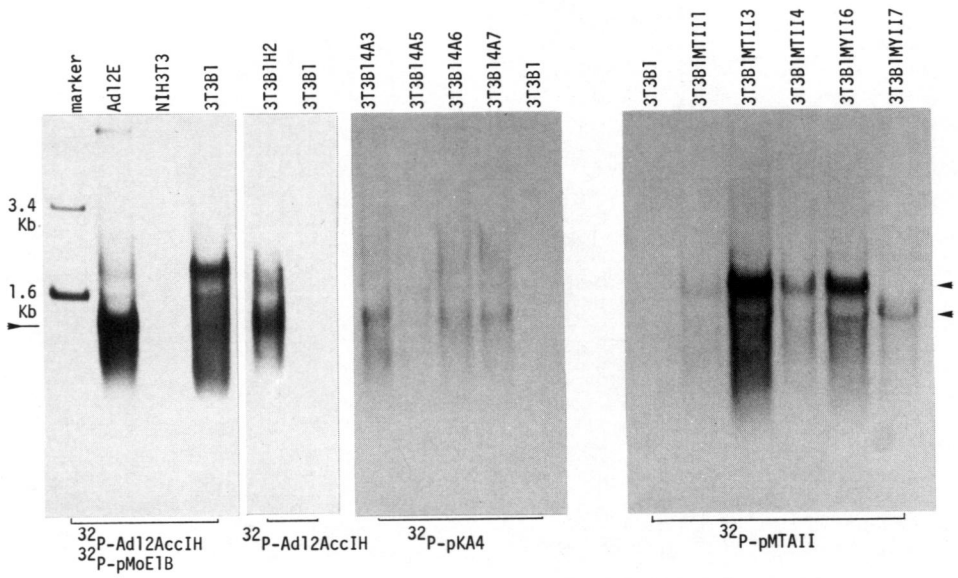


**Fig. 4** Northern blot hybridization of RNA from 3T3B1 cell lines transformed by p12AccH, pKA4 and pMTAII DNA. RNA blots (50 $\mu$g of total cytoplasmic RNA per lane) were hybridized with $^{32}$P-labeled viral DNA or recombinant plasmid DNA ($1\text{-}2\times10^{8}$ cpm/$\mu$g) and exposed to X-ray films for five days. Ad12E is cytoplasmic RNA (50 $\mu$g) prepared from KB cells early after lytic infection with Ad12. Arrows indicate adenovirus E1A mRNA (➛——) and major RNA bands ( ➛ ) specific to a SV40 DNA probe.

## DISCUSSION

Among twelve G418-resistant murine cell lines established by cotransfection with pSV2*neo* and pMoE1B containing Ad12 E1B with moloney murine sarcoma virus LTR, at least six cell lines contained detectable E1B mRNA. All of the six cell lines showed cell morphology similar to that of parental NIH3T3 cells, and reached saturation in monolayers and had no colony-forming ability in agar suspension cultures. These are compatible with the results obtained by van den Elsen *et*

*al.*(7) using primary and established rat cells, indicating that the Ad12 E1B gene has no detectable transforming activity in mouse NIH3T3 cells, too. On the other hand, cotransfection experiments showed that Ad12 E1B increased transforming efficiencies of p12AccH and pKA4 (more than a hundred fold) containing Ad12 E1A and Ad4 E1A, respectively, and a transforming efficiency of pMTAII (about two fold) carrying mutated SV40 early gene(5, 13, 23). When 3T3B1 cells carrying Ad12 E1B were transfected with pMTAII, the foci were induced more efficiently (five to eight fold) than in a case of cotransfection of NIH3T3 cells. Adenovirus mutants defective in the production of E1B 19k/21k peptides have undetectable or extremely decreased focus-forming activity(25-28). Our results also indicate that the Ad12 E1B has a strong enhancing activity of Ad12 and Ad4 E1A focus-formations. No detectable enhancing effect of Ad12 E1B on Ad5 E1A focus-formation was observed. Further studies should be carried out to investigate whether E1B polypeptide(s) is responsible for a transformation enhancing effect.

Viral (pPyLT and pMTAI) and cellular (c-*myc*) oncogenes which can immortalize primary rodent cells and/or transform primary cells cooperatively with an activated *ras* gene(24) did not induce focus-formation on 3T3B1 cell cultures. On the other hand, Ad12 E1B sequences can cooperate with or can enhance the transforming activity of the genes that have an incomplete transforming activity such as E1A and mutated SV40 early sequences. These raised the possibility of the 3T3B1 cell line carrying Ad12 E1B to be used for detection and isolation of the incomplete transforming sequences from cancer cell DNA in stead of NIH3T3 cells because of the enhancement of the focus-formation by incomplete transforming gene activities. The effects of Ad12 E1B on anchorage independency and tumorigenic properties are under investigation.

## ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research, for Special Project Research, Cancer-Bioscience, and for Scientific Research from the Ministry of Education, Science and Culture, Japan. This work was also supported in part by the Uehara Memorial Foundation Funds.

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