Establishment of a Rat Cell Line Carrying Hormone Inducible Human Adenovirus Type 12 E1A Gene

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SUMMARY

Human adenovirus type 12 (Ad12) E1A gene was cloned downstream of a hormone-inducible promoter of mouse mammary tumor virus (MMTV) to construct pME1A. In the transient expression assay, maximum CAT (chloramphenicol acetyltransferase) activity directed by MMTV LTR in the presence of dexamethasone (DX, 10^{-7} M) was less than 10% of that by Ad12 E1A promoter. On the other hand, focus-forming activity of pME1A was enhanced about sixty fold in rat 3Y1 cell culture in the presence of DX (10^{-6} M) and the resulting efficiency was in the same range as that of intact Ad12 E1A gene. A rat 3Y1 cell line YME4 carrying hormone-inducible Ad12 E1A gene was established from the transformed foci and used for further analyses. YME4 cells contained chimeric transcripts composed of MMTV LTR and Ad12 E1A sequences which became detectable within an hour after cells were exposed to DX (10^{-8} M). YME4 cells were transfected with several oncogenes for testing collaborative transformation by resident MMTV LTR-Ad12 E1A gene and additionally transfected oncogenes. Collaborative transformation of rat 3Y1 cells was observed by Ad12 E1A and v-*abl* oncogenes.

Key words: Adenovirus type 12 E1A gene, Mouse mammary tumor virus, Glucocorticoid hormone, Collaborative transformation, v-*abl*

INTRODUCTION

Transforming activity of human adenovirus resides in the left-end 11.2% of the viral genome, which contains two transcriptional units of early region 1A (E1A) and E1B(4). The viral DNA fragment carrying the E1A region alone cannot transform rodent cells to a potentially oncogenic state(8, 22), while it can immortalize unestablished rodent cells and render them susceptible to transformation by a second transforming gene such as adenovirus E1B(2), polyomavirus middle T(18) or human activated *ras* gene(20). These three genes can not induce transformation

in primary rodent cells when introduced alone(13, 18, 20, 29). Adenovirus E1A gene regulates the expression of other adenovirus early genes(1, 9) and cellular genes for heat shock protein(15), β-tubulin(24) or class I major histocompatibility antigens (21). However, the molecular mechanism in the process of transformation by adenovirus E1A gene including the relationship between the effect of the E1A gene on transcription and on immortalization has not yet been made clear.

In order to study the role of human adenovirus E1A gene in the process of transformation, we first constructed a recombinant plasmid containing human adenovirus type 12 (Ad12) E1A gene placed downstream of the hormone-inducible promoter of mouse mammary tumor virus (MMTV). A rat cell line in which the expression of Ad12 E1A gene was regulated by glucocorticoid hormone was established and analyzed. Collaborative cell transformation by hormone-induced Ad12 E1A gene and other transforming genes was tested by using rat cells carrying MMTV long terminal repeat (LTR)-Ad12 E1A gene.

MATERIALS AND METHODS

Plasmids

To construct a plasmid pME1A, the Ad12 E1A *Hae*III (442 nucleotide position [n. p.] from the left end of the viral genome)-*Acc*I (1594 n. p.) fragment, of which the left end was converted from *Hae*III to *Bam*HI site by *Bam*HI linker, was inserted between *Bam*HI and *Acc*I sites of MMTV-LTR carrying plasmid p5031 (Fig. 1). p5031, provided by Dr. Harold Varmus, contains LTR sequence of 960 base pairs (bp) (−863 to +90 n. p. from transcriptional start [cap] site) derived from MMTV proviral sequence which was inserted between *Cla*I and *Bam*HI sites of pBR322(14). Plasmid pMoE1A and pHSIE1A contain the Ad12 E1A *Hae*III fragment (442-1702 n. p.) at the *Bam*HI site of pMoLTR and pHSI, respectively (Fig. 1). pMoLTR carries LTR sequence (−444 to +28 n. p. from cap site) of Molony murine sarcoma virus (MoMSV) between *Eco*RI and *Sma*I sites of pUC8 (28). pHSI is a plasmid carrying human metallothionein II (hMT-IIA) promoter (−770 to +70 n. p. from cap site) at *Eco*RI site of pUC8 and was provided by Dr. Michael Karin(10). A plasmid p12E1ACAT carries Ad12 E1A transcriptional control sequence placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. The clone was constructed by inserting the Ad12 left-end *Hin*dIII-*Nar*I fragment (0-442 n. p.) between the *Hin*dIII and *Acc*I sites of pUCCAT. The pUCCAT, was provided by Dr. G. Chinnadurai, constructed from pSV2CAT(5) by subcloning the *Sma*I-*Eco*RI fragment which carries CAT-coding, SV40-splicing and SV40 poly(A)-addition sequences into the *Sma*I-*Eco*RI site of pUC13. A plasmid pMMLTRCAT carries a 1.0-kb *Eco*RI-*Bam*HI fragment of MMTV-LTR sequence that was prepared from p5031 and integrated into the *Sma*I site of pUCCAT after

both ends of the fragment blunted by using DNA polymerase I (large fragment of Klenow, Boehringer Manheim Corporation). Plasmid p12AccH carries Ad12 *Acc*IH fragment (0-1594 n. p.) which contains a whole E1A region integrated between *Eco*RI and *Acc*I sites of pBR322(28). pPyMT is a plasmid carrying a polyomavirus-middle-T coding sequence(18). pAM is a plasmid carrying the whole proviral sequence of Abelson murine leukemia virus at the *Eco*RI site of pBR322(23) and was provided by Dr. Nobuo Tsuchida. Transformation of *Escherichia coli* HB101, and propagation and purification of plasmid DNAs were performed as described previously(27). Restriciton enzymes, synthetic linkers and T4 ligase were purchased from Takara Shuzo Co. Ltd.

Cell culture and DNA transfection

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS, Flow Laboratories). For DNA transfection rat 3Y1 cells(11) were seeded at 5×10^5 cells per 6 cm dish and DNA was introduced into the cells at 24 hr after seeding by calcium phosphate precipitation as described(6). Transfected cell cultures were treated with 25% dimethyl sulfoxide at four hours after DNA inoculation, re-fed with fresh media and cultured in DMEM with 5% FBS in the presence or absence of dexamethasone DX (dexamethasone sodium phosphate, Merck and Co. Inc.).

Colony formation in soft agar

A single cell suspension in 4 m*l* (6 cm dish) or 8 m*l* (10 cm dish) of 0.3% agar (Agar purified, Difco Laboratories) was overlayed onto 6 m*l* (6 cm dish) or 10 m*l* (10 cm dish) of 0.5% agar base. Agar suspensions were made in DMEM with 10% FBS.

CAT assay

Cell extracts were made 40 hrs after transfection and CAT activity was measured as described(5). Briefly, the cell extracts were incubated with ^{14}C-chloramphenicol and 4 mM acetyl-CoA (Pharmacia) in 250 mM Tris-HCl (pH 7.8) at 37℃ for 30 min. After analysis by ascending thin layer chromatography, the conversion rates were determined by scintillation counting.

Southern and Northern blot hybridization

Preparation of large-molecular-weight cell DNA and cytoplasmic RNA from cultured cells, agarose gel electrophoresis and transfer to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories) have been described previously(26). Probes were labeled with ^{32}P by using nick translation kit (Boehringer

Manheim Corporation) with specific activities: $1\text{-}2\times10^8$ cpm/μg. For detecion of Ad12 E1A, MMTV-LTR and v-*abl* sequences, we used ^{32}P-labeled Ad12 E1A probe prepared from 1.6-kb *Eco*RI-*Acc*I fragment of p12AccH, MMTV-LTR probe from 1.0-kb *Cla*I-*Bam*HI fragment of p5031 and 5.1-kb *Kpn*I fragment of pAM, respectively. Southern and Northern blot hybridization procedures were performed as described before(26).

RESULTS

Transforming activity of recombinant DNAs

We constructed p12E1ACAT and pMMLTRCAT in which Ad12 E1A promoter and MMTV LTR were placed upstream of the coding sequence of CAT gene,

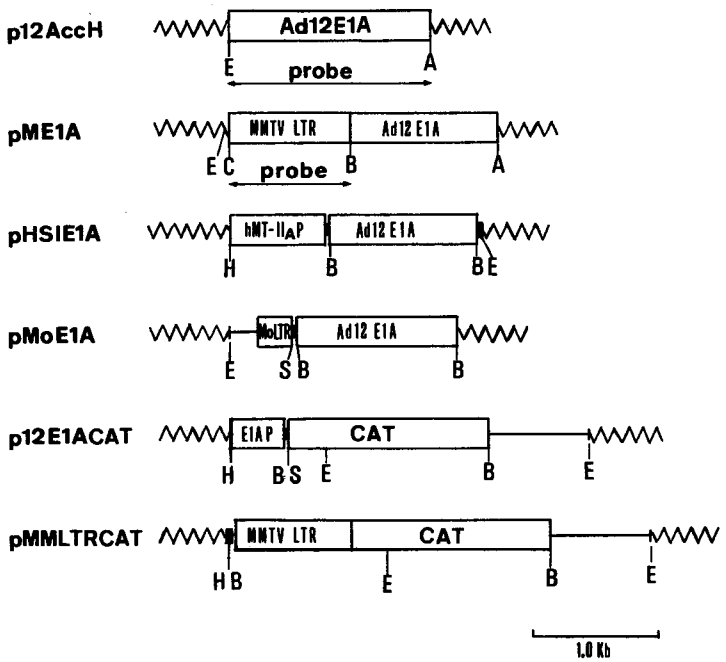

Fig. 1 Schematic representation of recmbinant plasmids used in transformation and CAT assay. p12AccH encodes the complete Ad12 E1A gene in Ad12 AccIH fragment (0-1594 n. p.)[28]. pME1A carries Ad12 E1A coding region (442-1594 n. p.) linked to upstream MMTV LTR. pHSIE1A and pMoE1A carries Ad12 E1A coding region plus N-terminal segment of E1B (442—1702 n. p.)[13] placed downstream of hMT-IIA promoter and MoMSV LTR, respectively. p12E1ACAT and pMMLTRCAT were derived by inserting the Ad12 E1A upstream region (0—442 n. p.) and MMTV LTR (−860 to +103 from transcriptional start site) upstream of coding sequence of CAT gene, respectively. The restriction enzyme sites indicated are: A; *Acc*I, B; *Bam*HI, C; *Cla*I, E; *Eco*RI, S; *Sma*I.
(∼∼∼); plasmid sequences, (——); viral or cellular sequences, (■); polylinker sequences.

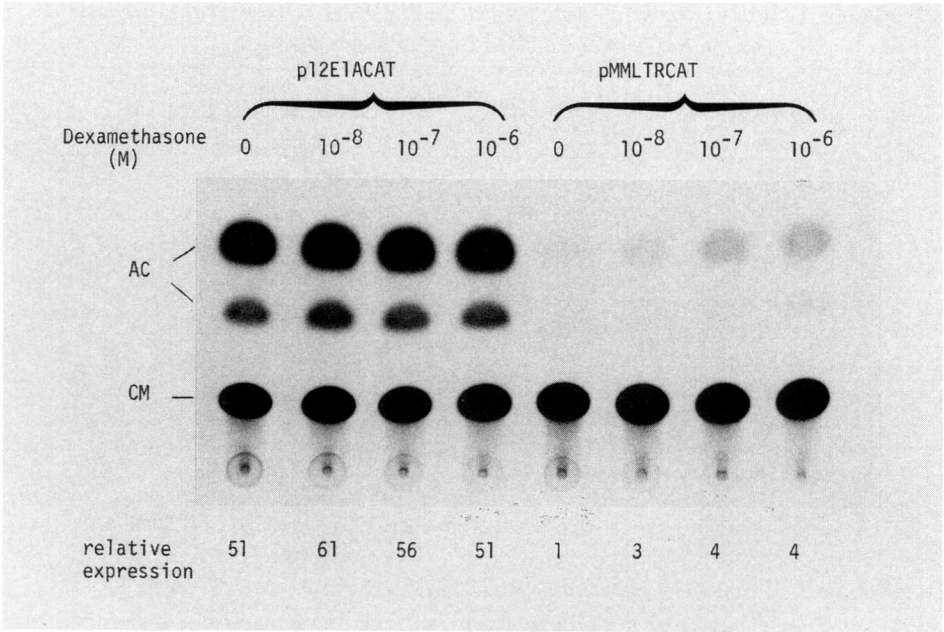

Fig. 2 Relative expression of CAT in rat 3Y1 cells transfected with p12E1A CAT and pMMLTRCAT in the presence or absence of dexamethasone. The value for the transfection of pMMLTR CAT wihtout hormone is arbitarily set to 1.0.

respectively (Fig. 1). The transient gene expression assay in rat 3Y1 cells showed that MMTV LTR-directed CAT activity was enhanced approximately four fold by DX (10^{-7} M) (Fig. 2). However, the maximum CAT activity under the control of MMTV LTR in the presence of DX was less than 10% of that under the Ad12 E1A promoter (Fig. 2)

Rat 3Y1 cells were transfected with recombinant plasmids carrying Ad12 E1A gene with various promoters to examine their focus-forming activities in the presence or absence of DX (10^{-6} M) (Table 1). Relative focus-forming activites were as follows: pMoE1A (DX) 380, pMoE1A 318, pHSIE1A (DX) 120, pHSIE1A 79, p12AccH 75, pME1A (DX) 63, p12AccH (DX) 50 and pME1A 1. Apart from transient gene expression study, focus-forming efficiency by pME1A was increased in the DX-containing media by appoximately sixty fold and the enhanced efficiency was in the same range of that by intact Ad12 E1A gene (p12AccH). hMT-IIA promoter also contains steroid-inducible transcriptional elements(10). However, pHSIE1A induced transformed foci with an efficiency about same as p12AccH even in the DX free media, and only a slightly enhanced focus-formation (about 1.5 fold) was observed in the DX-containing media.

Table 1 *Focus-forming Activities of Ad12 E1A Recombinants in Rat 3Y1 Cells with or without Dexamethasone.*

Recombinat DNA[a]		μg genome equivalent/dish[b]	Number of foci[c] Dexamethasone(M) 0	10^{-6}
Exp. I	p12AccH	10.0	55, 70 (6.3)[d]	51, 53 (5.2)
	pME1A	10.0	1, 1 (0.1)	57, 60 (5.9)
	pHSIE1A	10.0	61, 69 (6.5)	115, 127 (12.1)
Exp. II	p12AccH	5.0	35, 46 (8.1)	23, 24 (4.7)
	pME1A	5.0	0, 1 (0.1)	31, 36 (6.7)
	pHSIE1A	5.0	45, 47 (9.2)	53, 65 (11.8)
	pMoE1A	2.0	58, 69 (31.8)	70, 82 (38.0)

a) See Fig. 1 for recombinant construction.
b) Plasmid DNA, equivalent to 2.0, 5.0 or 10.0 μg Ad12 genome per dish, was linearized by *Eco*RI, mixed with calf thymus DNA used as a carrier (total 5.0 μg/dish) and transfected into 3Y1 cell culture.
c) Number of foci were counted four weeks after transfection.
d) Numbers in the parenthesis were average numbers of foci per μg Ad12 genome.

Establishment of hormone-inducible Ad12 E1A carrying rat cell lines

More than twenty rat 3Y1 lines were isolated from each single transformed focus induced by pME1A in the presence of DX. These lines were evaluated in terms of saturation densities and levels of Ad12 E1A mRNA with or without hormone (data not shown). One of the lines, designated as YME4, had saturation density approximately five fold higher in the media with 5% FBS and DX and about eleven fold higher with 2% FBS and DX compared to the saturation density in each case without a hormone supplement (Table 2). YME4 cells had fibroblastoid morphology in DX free media similar to parental 3Y1 cells, while they showed as epithelioid form in the media with DX (Fig. 3). Southern blot hybridization

Table 2 *Saturation Densities of 3Y1 and YME4 Cells in the Presence or Absence of Dexamethasone.*

Cells	FBS (%)	Dexamethasone (M)	Saturation densities[a] (cells/cm²)	Ratio (10^{-7}/0 M)
3Y1	5	0	0.60×10^5	1.3
	〃	10^{-7}	0.75×10^5	
	2	0	0.39×10^5	1.0
	〃	10^{-7}	0.40×10^5	
YME4	5	0	1.50×10^5	4.1
	〃	10^{-7}	6.16×10^5	
	2	0	0.52×10^5	11.0
	〃	10^{-7}	5.70×10^5	

a) Cells were seeded at 10^5/6 cm dish and cultured for three weeks in the media with or without dexamethasone. Saturation densities were deduced from growth curves.

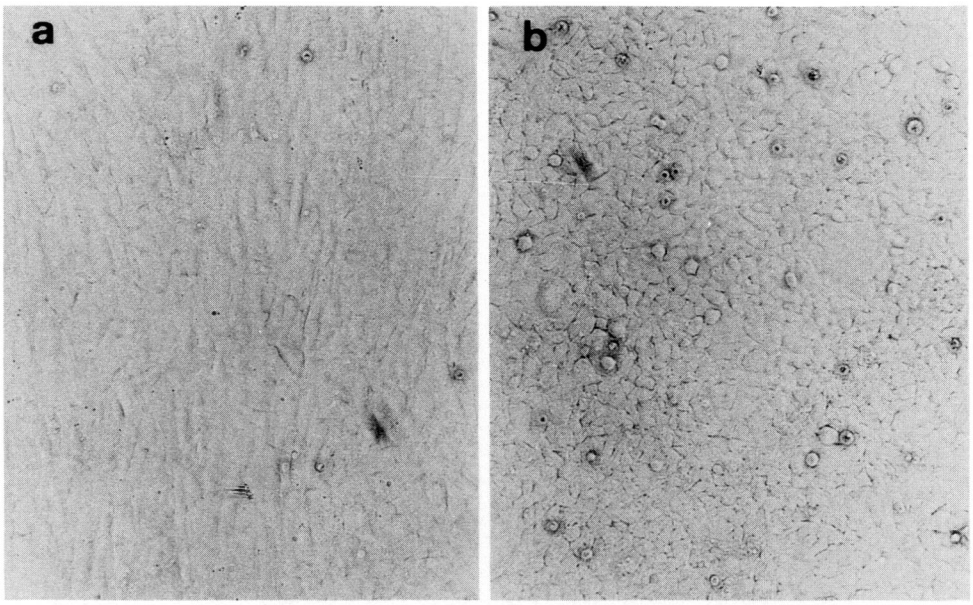

Fig. 3 Morphology of cultured YME4 cells. 5×10^5 cells per 6 cm dish were seeded and cultured for a week in the media with 5% FBS (a) or with 5% FBS and 10^{-7} M of dexamethasone (b). Magnification: 100×

revealed the presence of multiple copies of Ad12 E1A sequences in YME4 cell DNA (Fig. 4). Northern blot hybridization detected about 1.0-kb Ad12 E1A mRNA in the cytoplasmic RNA prepared from YME4 cells cultured in the DX media (Fig. 4, B). The same sized RNA bands were demonstrated in the RNA blots of YME4 cells hybridized to the MMTV LTR (Fig. 4, B), suggesting that the viral mRNA was composed of fused MMTV LTR and Ad12 E1A sequencees. The Ad12 E1A transcript became detectable within an hour after cells were exposed to DX and they became detectable in RNA blots with more than 10^{-8} M of DX (Fig. 5).

Collaborative transformation by Ad12 E1A and v-abl

Since YME4 cells form no colonies in 0.3% agar media with DX, we tested to see whether complete transformation of YME4 cells could occur by steroid-induced Ad12 E1A gene integrated and another transforming gene. YME4 cells were transfected with pPyMT (polyomavirus middle T gene) or pAM (v-*abl*) DNA followed by an incubation in 0.3% agar with or without DX (10^{-7} M) (Table 3). YME4 cells transfected with v-*abl* formed colonies in DX-containing agar with sizes and numbers larger than those in DX-free agar, suggesting the cooperative or enhanced transformation by Ad12 E1A and v-*abl* genes or by a v-*abl* gene and glucocorticoid hormone.

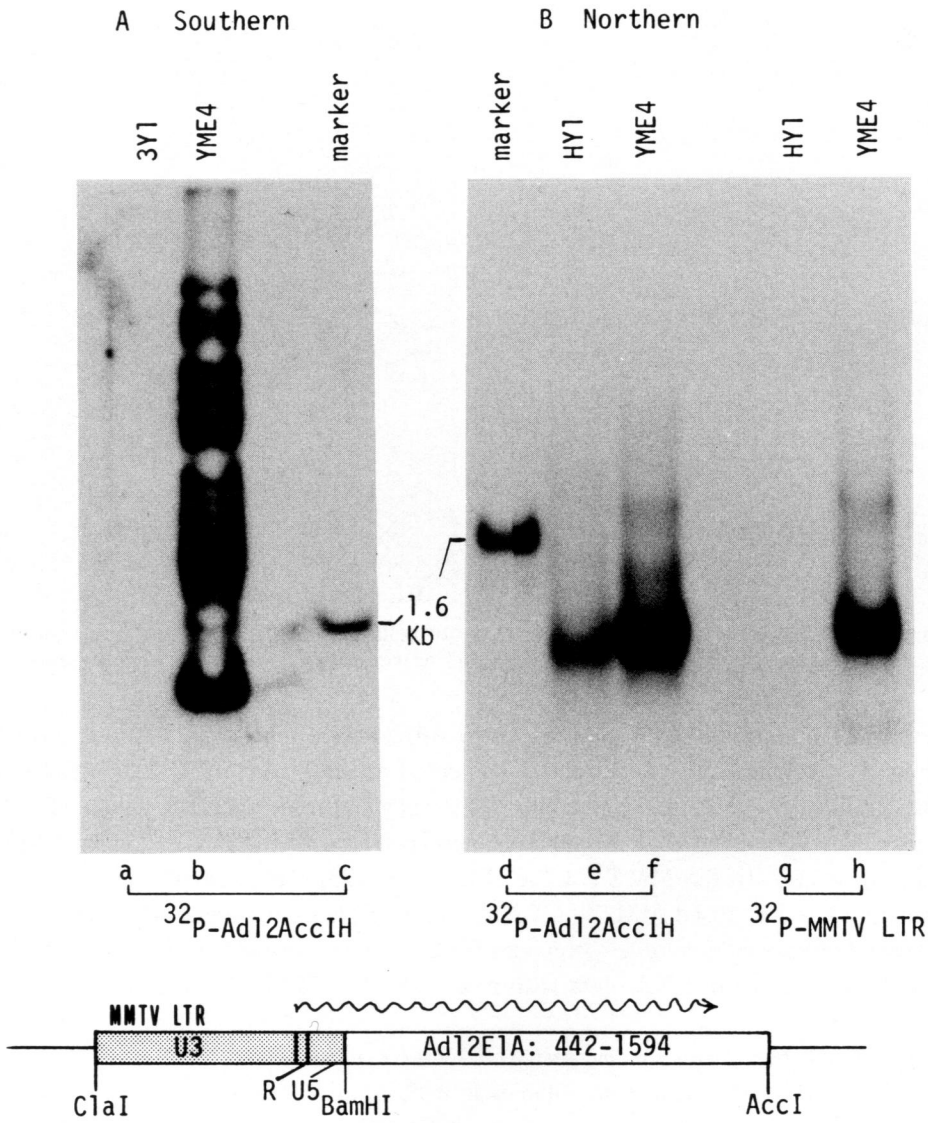

Fig. 4 Viral DNA and RNA analyses in YME4 cells. (A) For Southern blot analysis, 15 μg of cellular DNA was cleaved with *Bam*HI and *Acc*I, electrophoresed in 0.9% agarose gels and transferred to a nitrocellulose membrane. (B) For Northern blot analysis, RNA was prepared from YME4 cells cultured in the DX (10^{-7} M)-containing media and from HY1 cells (3Y1 line transformed by Ad12 AccIH fragment[22]) in normal media. Five μg (e, f) or 15 μg (g, h) of poly(A) RNA was electrophoresed in a 1.2% formaldehyde-agarose gel and transferred to a nitrocellulose membrane. DNA or RNA blots were hybridized to ^{32}P-labeled DNA fragment of Ad12 E1A (a-f) or MMTV LTR (g, h), washed and exposed to X-ray films for five days.

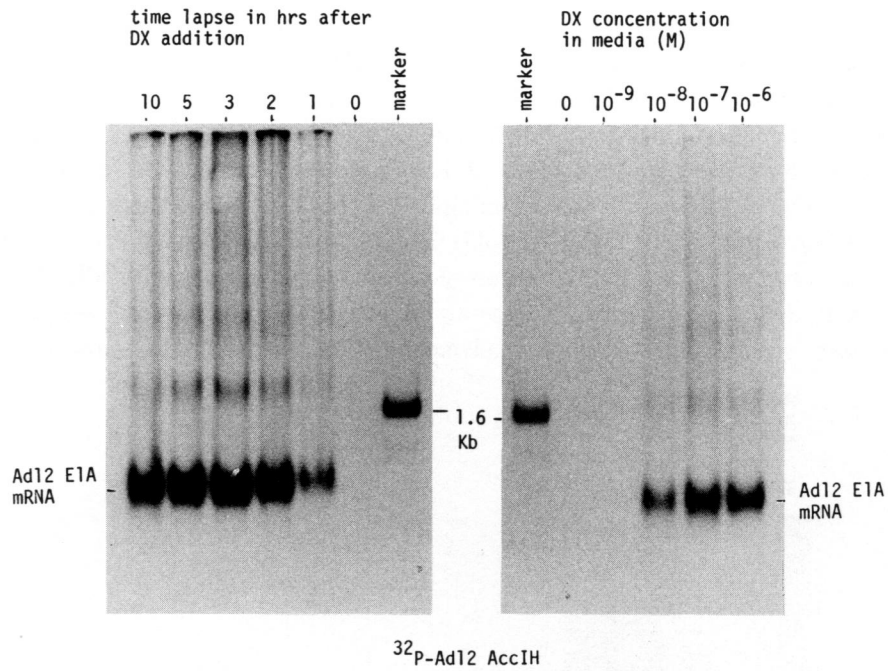

Fig. 5 Induction of MMTV LTR-Ad12 E1A gene in YME4 cells by dexamethasone. (A) YME4 cells reaching a confluent state was re-fed with fresh media with dexamethasone (10^{-7} M) and cytoplasmic RNA was prepared at one to ten hours after refeeding. (B) cytoplasmic RNA was prepared from cells cultured in the media with or without dexamethasone for 24 hrs. 50 μg of cytoplasmic RNA per lane was electrophoresed in 1.2% formaldehyde -agarose gels and transferred to nitrocellulose membranes. RNA blots were hybridized to ^{32}P-labeled DNA fragment of Ad12 E1A, washed and exposed to X-ray films for five days.

Table 3 *Colony-forming Efficiencies of 3Y1 and YME4 cells Transfected with Viral Oncogenes*[a].

Cell	DNA	μg/dish	Number of colonies/dish Dexamethasone (M)	
			0	10^{-7}
3Y1	calf thymus	5.0	0, 0	0, 0
	pPyMT	5.0	8, 10	25, 31
	p12AccH	5.0	0, 0	0, 0
	pAM	5.0	0, 1	5, 6
	p12AccH+pAM	5.0+5.0	8, 8	N. D.[b]
YME4	calf thymus	5.0	0, 0	0, 0
	pPyMT	5.0	118, 124	52, 70
	pAM	5.0	25, 37[c]	89, 91[d]

a) Cells transfected with calf thymus or plasmid DNA were detached 24 hrs after transfection and a single cell suspension was incubated for four weeks. See Fig. 1 for recombinant construction. b) not done
c) diameter: 0.2−0.5 mm d) diameter: 0.2−0.5mm (60%) and >0.5 mm (40%)

The cotransfection experiment was carried out by transfecting parental rat 3Y1 cells with a mixture of pAM and p12AccH (Table 3). As a result efficient colony formation was observed in 3Y1 cells transfected with both of these two transforming genes. All of the four lines isolated from colonies formed by pAM and p12AccH contained both the viral transcripts of 1.0-kb Ad12 E1A and about 5.0-kb v-*abl* mRNA (Fig. 6). It was also found that glucocorticoid hormone posesses a transformation enhancing effect (about ten fold) in the transformation by v-*abl* (Table 3). On the other hand, YME4 cells transfected with polyomavirus middle T gene (pPyMT) formed colonies in DX-containing agar with a frequency lower than in the case without a hormone supplement (Table 3). Since the transforming

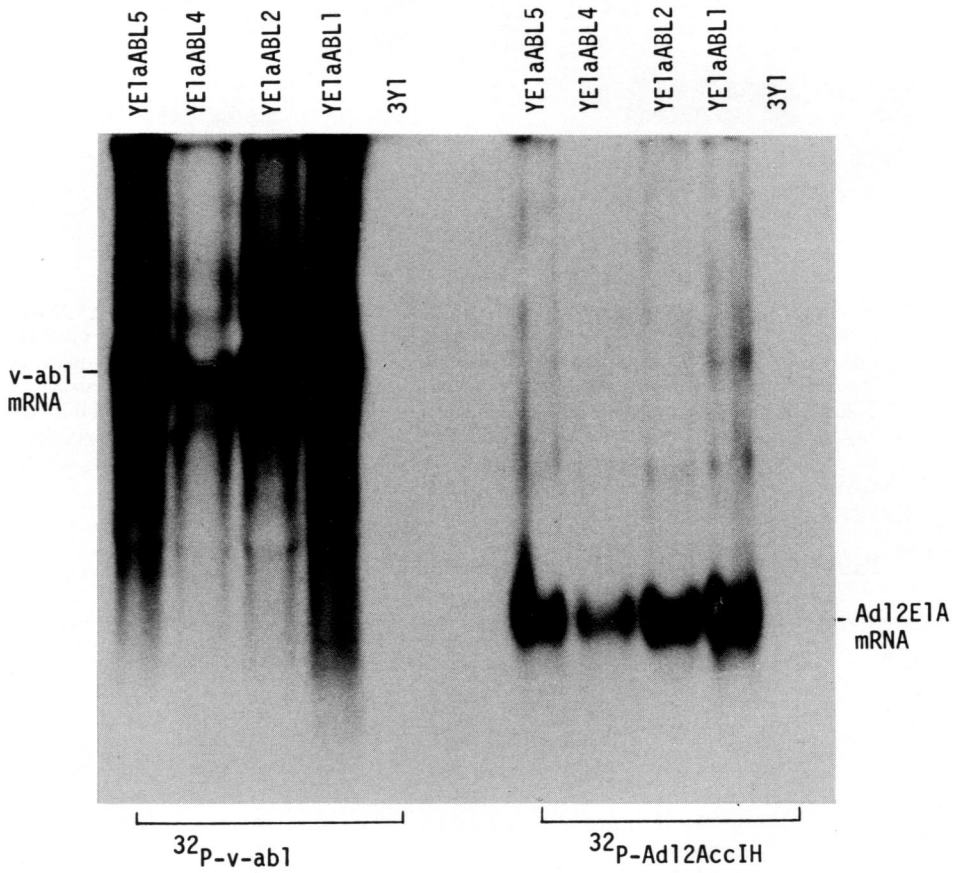

Fig. 6 Viral transcripts in 3Y1 lines, YE1aABL1, YE1aABL2, YE1aABL4 and YE1aABL5, transformed by co-transfection with Ad12 E1A and v-*abl*. 50 μg of cytoplasmic RNA was electrophoresed in 1.2% formaldehyde-agarose gels and transferred to a nitrocellulose membrane. RNA blots were hybridized to ^{32}P-labeled DNA fragment of v-*abl*(a) or Ad12 E1A(b) and exposed to X-ray films for five days.

efficiency in 3Y1 by pPyMT DNA was increased by two to three fold in soft agar with hormone (Table 3), it was suggested that a reduced colony-forming efficiency would result from the interaction between induced ad12 E1A and introduced polyomavirus middle T gene.

DISCUSSION

Recombinant plasmid carrying Ad12 E1A gene placed dewnstream of MMTV LTR (pME1A), hMT-IIA promoter (pHSIE1A) and MoMSV LTR (pMoE1A) were constructed and their focus-forming activities were examined by transfecting rat 3Y1 cells in the presence or absence of glucocorticoid hormone. pHSIE1A transformed rat 3Y1 cells with an efficiency in the same range as intact Ad12 E1A gene (p12AccH) even without the hormone. On the other hand, MMTV LTR showed a low level of basal transcription and efficient hormone-induced expression of genes downstream of MMTV LTR revealed from the study of focus-formation by pME1A and CAT assay under the control of MMTV LTR. From these results, it was confirmed that MMTV LTR was suitable for switching on and off the downstream genes especially when the products were toxic or showed transforming activities.

We established a rat cell line (YME4) carrying MMTV LTR-Ad12 E1A gene in which the expression was regualated by glucocorticoid hormone. In addition to the rapid induction of YME4-carrying MMTV LTR-Ad12 E1A gene (within an hour after exposure of cells to dexamethasone), YME4 cells showed incompletely transformed phenotypes in the media with dexamethasone with epithelioid morphology and high saturation density without colony-forming ability in soft agar.

Induction of cellular DNA synthesis by Ad12 E1A gene was studied by use of hormone-inducible promoter of MMTV LTR(18). In this paper, we used YME4 cells to test collaborative transformation by hormone-induced Ad12 E1A gene and transfected second oncogenes. As a result, collaborative transformation of rat 3Y1 cells with v-*abl* and Ad12 E1A genes was observed.

YME4 cells transfected with polyomavirus middle T gene (pPyMT) formed reduced colonies in hormone-containing agar. This effect was also found in YME4 sublines carrying pPyMT sequences established from G418-resistant colonies after cells were transfected with pPyMT and pSV2*neo* (data not shown). Since glucocorticoid hormone increased transforming efficiency in rat 3Y1 cells by pPyMT, it was suggested that decreased transformation efficiency of pPyMT-carrying YME4 cells was a result of the transregulation (transsuppression) effect of Ad12 E1A gene on polyomavirus early promoter(3). Glucocorticoid hormone enhances transformation by polyomavirus(17), by herpes simplex virus type 2(7) and by Kirsten strain of mouse sarcoma virus (Ki MSV)(19). In addition to

collaborative transformation of 3Y1 cells by v-*abl* and Ad12 E1A gene, we also found that dexamethasone itself enhances transformation of 3Y1 cells by v-*abl*.

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