CELL STRUCTURE AND FUNCTION 18: 457–465 (1993)

Isolation of Flat Revertants from Human Papillomavirus Type 18 E6E7 Transformed 3Y1 Cells by Transfection with a Rat Embryo Fibroblast cDNA Expression Library

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Key words: human papillomavirus/transformation/cDNA expression library/revertants

ABSTRACT. **A rat embryo fibroblast (REF) cDNA expression library was transfected into 3Y1 cells transformed by human papillomavirus type 18 E6 and E7 genes and 10 flat revertants were isolated. These revertants expressed the same levels of E6 and E7 mRNA as the parent cells, but had greatly reduced ability to form colonies in soft agar. Suppression of transformation was dominant in cell hybrids generated by fusing each revertant with the parental transformed cells. Furthermore, loss of transfected cDNA was observed in re-transformed cell hybrids derived from one flat revertant. Overexpression of the cDNA suppresses the colony-forming efficiency of the cells transformed by E6 and E7 genes.**

Human papillomavirus (HPV) types 16 and 18 have frequently been found in cervical carcinomas and are believed to play an important role in carcinogenesis. The E6 and E7 open reading frames are selectively retained and expressed in these cancer cells. These genes are also essential for transformation of established rodent cell lines in vitro. In mouse Ψ2 cells, E7 induces colony formation in soft agar and increases the saturation density, while E6 confers tumorigenicity in nude mice (29). In rat 3Y1 cells, E7 induces anchorage-independent growth (15, 25), and E6 enhances this function (11, 26). On the other hand, in primary cultured cells, E6 and E7 induce immortalization but not transformation (12). This difference might be due to divergence of the genetic background.

We have reported that the non-transformed phenotype is dominant in somatic cell hybrids between E6E7 transformed F2408 cells and primary rat embryo fibroblast (REF) (18). Therefore, REF may have genes that can suppress transformation by E6 and E7. To obtain these genes, we constructed a REF cDNA library with the pcD2 expression vector, and transfected it into E6E7 transformed 3Y1 cells. After treatment with cis-hydroxy-L-proline (CHP), which is preferentially toxic to transformed cells, ten independent flat revertants were isolated.

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MATERIALS AND METHODS

Cells. Samples of 5×10^5 3Y1 cells were transfected with 20 μg of p1571 (expressing a region (nt100-931) of HPV18 E6 and E7 under the human β actin promoter) and 2 μg of pHyg. Cells were selectively cultured with hygromycin (200 μg/ml) for 7 days, and surviving cells were cultured in 0.33% agar medium. After 6 weeks, several visible colonies were picked and cloned. One of these clones, 18T3Y1, shows a transformed morphology and has the ability to form colonies in soft agar.

18T3Y1-neo and 3Y1-neo were derived from 18T3Y1 and 3Y1, respectively, by transfection of pSV2neo and G418 selection (400 μg/ml). 18T3Y1-bsr was obtained by transfection with pSV2bsr (14) and selection with brasticidin-S (2 μg/ml).

cis-hydroxy-L-proline treatment. cis-hydroxy-L-proline (CHP) was obtained from Calbiochem. Boehring. 18T3Y1 and 3Y1 cells were plated at 1×10^4 cells per 10 cm dish in Dulbecco's modified minimum essential medium (DMEM) with 10% fetal calf serum. After one day, the medium was changed to fresh medium, the same as that used above plus 0–200 μg/ml CHP, and incubation was continued for 7 days. The medium was then changed to that without CHP, and after 7 days, the cells were fixed with 50% methanol and stained with Giemza solution. Then, surviving colonies were counted.

Transfection with a cDNA expression library. A REF cDNA library was constructed with rat embryo fibroblast mRNA and the expression vector pcD2 (3) by the Okayama-Berg method. Samples of 5×10^5 18T3Y1 cells were trans-

fected with 20 μg of the REF cDNA library by the calcium phosphate method modified by Chen & Okayama (3). After transfection, the cells were plated in ten dishes and selected with G418 (400 μg/ml) for 7 days. Surviving cells in each dish were pooled and 1×10^4 cells derived from each pool were plated in three or four dishes. After incubation for one day, the cells were treated with CHP (150 μg/ml) as described above. Surviving colonies were isolated using a cloning cylinder.

Northern blot hybridization. RNA was isolated from cells by the guanidium isothiocyanate-cesium chloride method (4). Samples containing 30 μg of total RNA were subjected to electrophoresis in a formaldehyde agarose gel and transferred to nylon filters. The filters were hybridized at 41° overnight in a solution of 50% formamide, 0.6 *M* NaCl, 60 m*M* sodium citrate, 0.2% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 50 μg /ml of herring sperm DNA with a labeled DNA probe. The hybridized filter was washed with 15 m*M* NaCl, 1.5 m*M* sodium citrate, 0.1% SDS at 50° and autoradiographed. The HindIII-HindIII fragment containing the E6 and E7 genes from p1571 was used as a probe. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plasmid was obtained from ATCC®.

Southern blot hybridization. Isolation of cellular high-molecular-weight DNA and DNA blot hybridization were performed as described (1, 23). DNA from each sample was digested with a restriction enzyme, separated by agarose gel electrophoresis, and transferred to a nylon filter. Hybridization and washing were done as described for Northern blotting.

Soft agar assay. Samples of 1×10^4 cells were inoculated into 0.33% agarose containing DMEM with 5% FCS in 6-cm dishes. After incubation for 3 weeks, colonies of more than 0.125 mm diameter were scored.

Somatic cell hybrids. Samples of 1×10^5 revertant cells and 1×10^5 18T3Y1-bsr were mixed and plated in 3-cm dishes. After incubation for one day, the cells were fused by treatment with polyethylene glycol for 1 min. From the next day, fused cells were selectively cultured with G418 (400 μg/ml) and brasticidin-S (2 μg/ml) in 10-cm dishes.

Recovery of cDNA plasmids. Samples containing 5 μg of genomic DNA of revertant cells were digested with the restriction enzyme SalI. The DNA digests were diluted with 400 μl of a ligation buffer (50 m*M* Tris-HCl pH 8.0, 10 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP) and ligated with 400 units of T4DNA ligase (Takara®) overnight. The ligated DNAs were precipitated with ethanol, rinsed and dissolved in 4 μl of distilled water. E. coli strain DH10B was transformed with 2 μl of DNA solution by electroporation and plated on LB-agar containing 100 μg/ml ampicillin. Colony hybridization was performed as described (16) with the neo-gene fragment used as a probe in Southern blotting. Similar experiments were done with pairs of the three enzymes NheI, SpeI and XbaI instead of SalI.

Plasmids. A 2.5 kb fragment that contains the SV40 promoter, neo resistance gene and polyadenylation signal, was derived from pMSG-CMV by digestion with BamHI. pSRa-neo was constructed by blunt-end ligation of this fragment into a HindIII site of pcDL-SRα296 (24). The 1.6 kb BamHI-BamHI fragment that contains N31 cDNA insert and the 1.0 kb BamHI-SphI fragment that deletes the 3′ region of N31 cDNA were ligated to 5.8 kb vector DNA derived from pSRa-neo by digestion with BamHI.

RESULTS

Effect of CHP on the survival of 3Y1 and 18T3Y1 cells. 18T3Y1 cells are 3Y1 cells transformed by HPV18 E6 and E7. A procedure for selection of non-transformed cells from transformed cells was necessary to obtain revertants from 18T3Y1 cells. Thus, we investigated the effects of cis-hydroxy-L-proline (CHP), which is preferentially toxic to transformed cells (5), on 3Y1 and 18T3Y1 cells. Revertant cells have been isolated from ras-transformed NIH3T3 cells with this agent (27).

18T3Y1 and 3Y1 cells were exposed to CHP at concentrations of 0–200 μg/ml and surviving colonies were counted. 18T3Y1 cells were more sensitive than 3Y1 cells to CHP (Table I). Using CHP with concentrations of 150 μg/ml or more, 18T3Y1 cells were completely killed. On the other hand, 20% and 6% of the nontransformed 3Y1 cells survived on treatments with CHP of 150 μg/ml and 200 μg/ml, respectively. Therefore 150 μg/ml of CHP was considered suitable for selection of non-transformed revertants from transformed 18T3Y1 cell.

Isolation of flat revertant cells. 18T3Y1 cells were transfected with the REF cDNA expression library, and after G418 selection, transfected cells were exposed to 150 μg/ml of CHP. Thirty-three flat clones were isolated from surviving colonies with a cloning cylinder. For evaluation of the expressions of the E6 and E7 genes, RNAs were extracted from these flat cell clones and analyzed by Northern blot hybridization (Fig. 1). Eighteen clones expressed the same levels of E6 and E7 mRNAs as the parental 18T3Y1 cells (lanes 2, 5, 6, 8, 9, 10, 13, 18, 19, 20, 21, 22, 23, 24, 26, 28, 29 and 36).

Table I. EFFECT OF CHP ON THE SURVIVAL OF 3Y1 AND 18T3Y1 CELLS

CHP concentration (μg/ml)	Number of surviving colonies[a]	
	3Y1	18T3Y1
0	324 (100%)	163 (100%)
50	374 (115%)	135 (82%)
75	388 (119%)	59 (36%)
100	246 (76%)	18 (11%)
150	64 (20%)	0 (0%)
200	18 (6%)	0 (0%)

[a] 10^4 cells were plated.

However, the expressions of E6 and E7 were not detected or were markedly reduced in 15 clones. The 18 clones that expressed the parental levels of E6 and E7 mRNAs were then cultured, but six clones were unstable, their morphology changing to that of 18T3Y1 cells. The other 12 clones that showed stable flat phenotype were analyzed further. For estimation of the numbers of cDNA copies, DNAs extracted from these 12 clones were digested with BamHI and analysed by Southern blot hybridization with a neo-gene as a probe (Fig. 2). The DNA used as a probe was the HindIII-BamHI fragment of pSV2neo containing the neo-gene and SV40 polyA site. As the SV40 polyA site is present for expression of the neo-gene and inserted cDNA in pcD2 vector, not only the neo-gene, but also the SV40 polyA site hybridized with this probe. The patterns of hybridized bands in R11 and R15 were identical (lanes 1 and 2). Since these clones were isolated from the same primary dish, they were thought to be siblings. For a similar reason, R51 and R54 were also thought to be siblings (lanes 6 and 7). Therefore, 10 independent revertant clones were seemingly isolated. We calculated the sum of the band densities in each lane determined by densitometry, and from the value, estimated the number of integrated cDNA plasmids. The results indicated that each revertant had 1–8 cDNA plasmids (Table II).

The morphological phenotypes of 18T3Y1 cells and the revertants are shown in Fig. 3. 18T3Y1 cells are small and slender, and show overlapping growth. The morphologies of the revertant cells varied, but all showed a stable flat phenotype and exhibited contact inhibition. 18T3Y1 cells showed 3–8% colony-forming efficiency in soft agar. The revertants all had markedly reduced ability for anchorage-independent growth (Table III). Thus, the morphology and growth in soft agar of the revertants showed non-transformed characters.

Dominance of the non-transformed phenotype in soft agar assay. Reversion might not be caused by expression of transfected cDNAs but by cellular alteration of transformed cells. To examine this possibility, we prepared somatic cell hybrids between revertants and 18T3Y1 cells (Table IV). The hybrid cells showed intermediate morphology or that of almost completely transformed cells. However, colony-forming ability in soft agar was suppressed by cell fusion. The colony-forming efficiency of hybrids between non-transformed 3Y1 and 18T3Y1 cells was not lower than that of 18T3Y1-18T3Y1 hybrids. Therefore, it is more likely that the dominant transformation suppression in revertants is

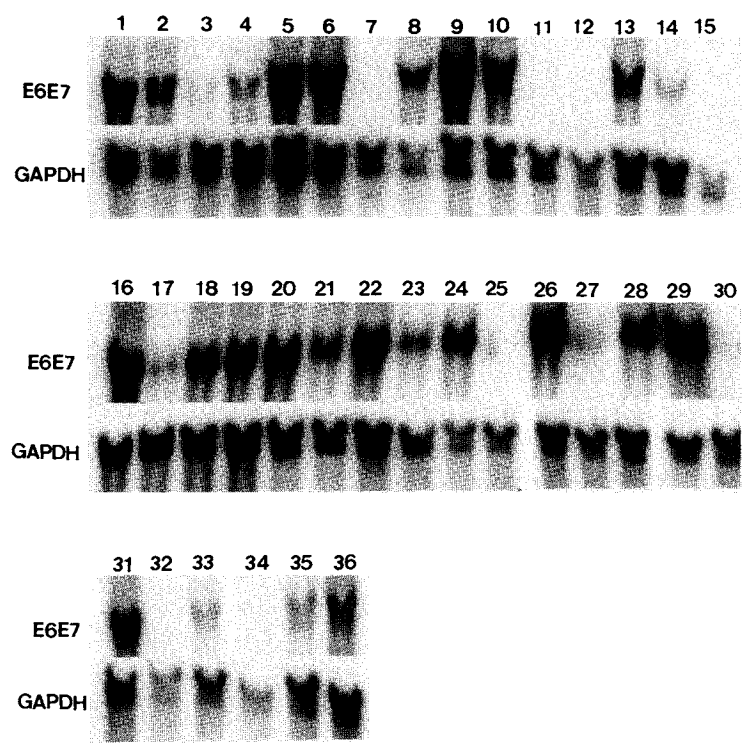

Fig. 1. Northern blots. Upper parts show the expression of the E6 and E7 genes in 18T3Y1 and flat clone cells. Lower parts show expression of GAPDH as a control. RNAs were from 18T3Y1 cells (lanes 1, 16 and 31) and flat clones (lanes 2–15, 17–30 and 32–36). Samples from revertants are shown in lane 2 (R11), lane 5 (R15), lane 9 (R31), lane 10 (R32), lane 13 (R41), lane 18 (R51), lane 19 (R54), lane 20 (R56), lane 21 (R62), lane 23 (R64), lane 26 (R74), and lane 28 (R77).

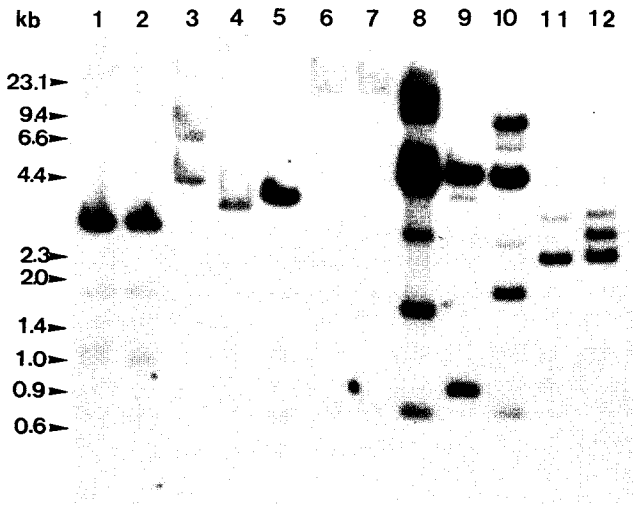

Fig. 2. Southern blot analysis. Samples of 15 μg of DNA extracted from revertants were digested with BamHI and analyzed by Southern blot hybridization. The BamHI-HindIII fragment of pSV2neo, which contains the neomycin resistance gene and SV40 polyA site, was used as a probe. DNA samples were from R11 (lane 1), R15 (lane 2), R31 (lane 3), R32 (lane 4), R41 (lane 5), R51 (lane 6), R54 (lane 7), R56 (lane 8), R62 (lane 9), R64 (lane 10), R74 (lane 11) and R77 (lane 12).

due to expression of transfected cDNAs than that reversion is a recessive cellular process.

Recovery of cDNA plasmids. We tried to recover pcD2 cDNA plasmids by reported methods (9, 20). Genomic DNA extracted from revertants was digested with SalI, which cuts the pcD2 vector at one site. The digested DNAs were circularized by self-ligation at low concentration of DNA and transformed E. coli efficiently on high-voltage electroporation. As not only pcD2 but also p1571 (HPV18 E6E7) and pHyg have an ampicillin-resistance gene, bacterial colonies transformed by circularized plasmid DNA derived from pcD2 were detected by the colony hybridization method using the neo-gene as a probe. Plasmid DNAs were prepared from positive colonies and digested with BamHI or XhoI, which cut the pcD2 vector at both ends of the inserted cDNA. Plasmids that showed the same or very similar digested patterns were regarded as identical. Only two and one plasmids were recovered from R56 and R64, respectively. No cDNA plasmid was recovered from other revertants. As SalI is an enzyme which cuts the pcD2 vector at one site, the plasmid can be recovered only when it is integrated into the host DNA in a tandem repeat form.

To recover the cDNA plasmids more efficiently, we modified the method. When an enzyme which dose not cut pcD2 is used, the plasmid may be recovered in a form containing host DNA even if it did not integrate into the host gene as a tandem repeat. The pcD2 vector has no site recognized by NheI, SpeI or XbaI, and the ends of a DNA fragment digested with these enzymes can be ligated to each other. The mean sizes of rodent genomic DNA fragments digested with NheI, SpeI and XbaI are 10 kb, 13 kb and 7 kb, respectively. The pcD2 vector is 4.6 kb and the mean length of inserted cDNA is about 1.5 kb. For circularization, the plasmid cannot exceed 15 kb in size, so pairs of the three enzymes were used. As shown in Table II, we recovered cDNA plasmids from R31, R62 and R74 with NheI, SpeI and XbaI but not with SalI. Recoveries from R56 and R64 were more efficient. However, no cDNA plasmid could be recovered from R11, R32, R41, R51 or R77.

Loss of transfected cDNA in re-transformed cell hybrids. R31 was estimated to contain two cDNA copies (Fig. 4). Two plasmids were recovered from this cell clone with XbaI-NheI or NheI-SpeI, but both plasmids have an insert of about 1.1 kb cDNA and are likely to have the same cDNA by the hybridization method. R31 is a stable revertant clone and forms very few, if any, colonies in soft agar. Hybrids between R31 and 18T3Y1 cells formed colonies in soft agar at a low rate

Table II. ESTIMATED NUMBERS OF cDNA COPIES AND NUMBERS OF RECOVERED cDNA PLASMIDS.

Cells	Estimated numbers[a] of cDNA copies	Numbers of cDNA plasmids recovered			
		SalI	SpeIXbaI	XbaINheI	NheISpeI
R11	3	0	0	0	0
R31	2	0	0	1	1
R32	1	0	0	0	0
R41	2	0	0	0	0
R51	1	0	0	0	0
R56	8	2	4	3	5
R62	3	0	0	2	1
R64	4	1	0	1	1
R74	2	0	0	1	0
R77	3	0	0	0	0

[a] Estimated from the sum of band densities in Fig. 2 determined by densitometry.

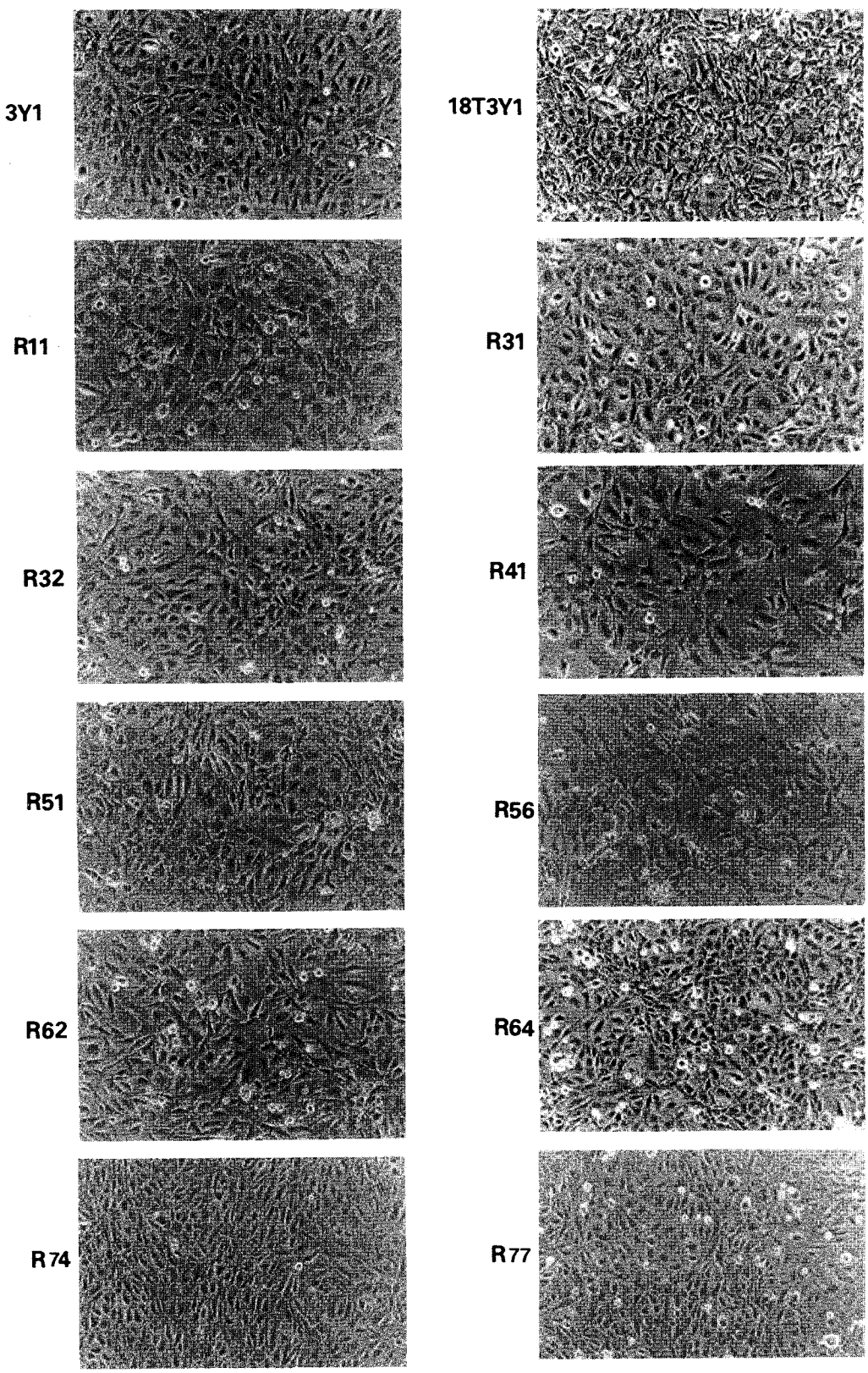

Fig. 3. Morphologies of 3Y1, 18T3Y1 and revertant cells. (×100)

Table III. SOFT AGAR ASSAY OF REVERTANT CELLS.

Cells	Colony formation in soft agar (%)[a]		
	Exp. 1	Exp. 2	Exp. 3
3Y1	0.00	—	—
18T3Y1	8.17	3.36	3.37
R11	—	0.00	—
R31	—	0.01	—
R32	—	—	0.00
R41	—	—	0.02
R51	—	—	0.07
R56	—	—	0.00
R62	—	—	0.05
R64	—	—	0.03
R74	—	0.07	—
R77	—	0.05	—

[a] Samples of 1×10^4 cells were incubated in 0.33% agarose medium for 3 weeks. Colonies larger than 0.125 mm in diameter were counted.

(Table IV). Some of these colonies were cloned, and the possibility of rearrangement of the transfected cDNA was examined. DNAs were extracted from five re-transformed hybrid cells (named 31RTH1-5), digested with BamHI or XhoI, and analyzed by Southern blot hybridization. A 1.1 kb PstI-BamHI fragment that contains a cDNA insert of the plasmid recovered with XbaI-NheI was used as a probe. As shown in Fig. 4, the 1.2 kb band in R31 (lanes 2 and 9) was lost in the re-transformed hybrid cells after digestion with either BamHI (lanes 3–7) or XhoI (lanes 10–14). Thus the loss of this cDNA may induce re-transformation. The bands of high molecular weights may be due to hybridization of the probe with the same cDNA repeated incompletely or to hybridization of a part derived from the vector of this probe with other transfected pcD2 plasmids.

Table IV. SOFT AGAR ASSAY OF CELL HYBRIDS BETWEEN REVERTANTS AND 18T3Y1 CELLS.

Cell hybrids	Colony formation in soft agar (%)[a]		
	Exp. 1	Exp. 2	Exp. 3
18T3Y1-bsr × 3Y1-neo	—	—	6.30
× 18T3Y1-neo	3.46	4.04	7.52
× R11	0.64	0.50	—
× R31	0.14	0.29	—
× R32	0.40	0.53	—
× R41	0.98	1.02	—
× R51	0.58	0.54	—
× R56	0.25	0.28	—
× R62	0.62	0.72	—
× R64	0.97	0.84	—
× R74	0.75	0.59	—
× R77	0.73	0.87	—

[a] Samples of 1×10^4 hybrid cells were incubated in 0.33% agarose medium for 3 weeks. Colonies larger than 0.125 mm in diameter were counted.

Overexpression of N31 suppresses the colony-forming efficiency of 18T3Y1 cells in soft agar. The plasmid recovered from R31 with XbaI and NheI (named p31XN7) was transfected to 18T3Y1 cells. However, the transfected cells showed no significant change in morphology and an anchorage-independent growth. We considered the possibility that the p31XN7 insert cDNA might be rearranged in plasmid recovery and that the expression of the cDNA might be insufficient. Therefore, the REF cDNA library was screened with p31XN7 insert cDNA as the probe, and the pcD2 plasmid which has about 1.5 kb cDNA insert was obtained (named N31). N31 and p31XN7 insert cDNAs were sequenced (19). The sequence of N31 cDNA was identified with p31XN7 insert cDNA in the 3′ overlapping region and was 395 bp longer at the 5′ end. The 3′ region of N31 contains one ATTTA motif that mediates mRNA degradation (22). To express cDNA more efficiently, N31 insert and a 1.0 kb fragment that deletes the 3′ region containing the ATTTA motif were ligated into pSRα-neo vector (named pSRαN31-neo and pSRαN31ΔSB-neo). pSRαN31-neo, pSRαN31ΔSB-neo, and pSRα-neo were transfected into 18T3Y1 cells. After the selective culture with G418, surviving colonies were isolated. The expression of N31 and the colony-forming efficiency in soft agar were examined in each clone. pSRαN31-neo-transfected clones expressed N31 insufficiently and had a minor change in the colony-forming efficiency in soft agar (data not shown). However, as

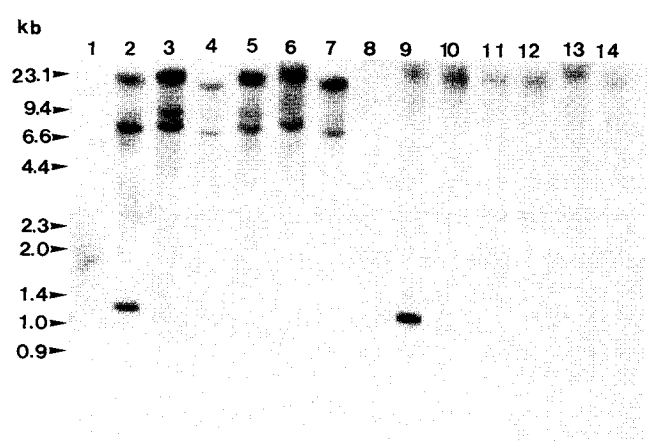

Fig. 4. Southern blot analysis of re-transformed hybrid cells. 20 μg of DNAs extracted from 18T3Y1, R31 and re-transformed hybrid cells (named 31RTH1-5) were digested with BamHI (lane 1–7) or XhoI (lane 8–14). A 1.1 kb PstI-BamHI fragment, which contains a cDNA insert of the plasmid recovered from R31 with XbaI-NheI, was used as a probe. DNA samples were from 18T3Y1 (lanes 1 and 8), R31 (lanes 2 and 9), 31RTH1 (lanes 3 and 10), 31RTH2 (lanes 4 and 11), 31RTH3 (lanes 5 and 12), 31RTH4 (lanes 6 and 13) and 31RTH5 (lane 7 and 14).

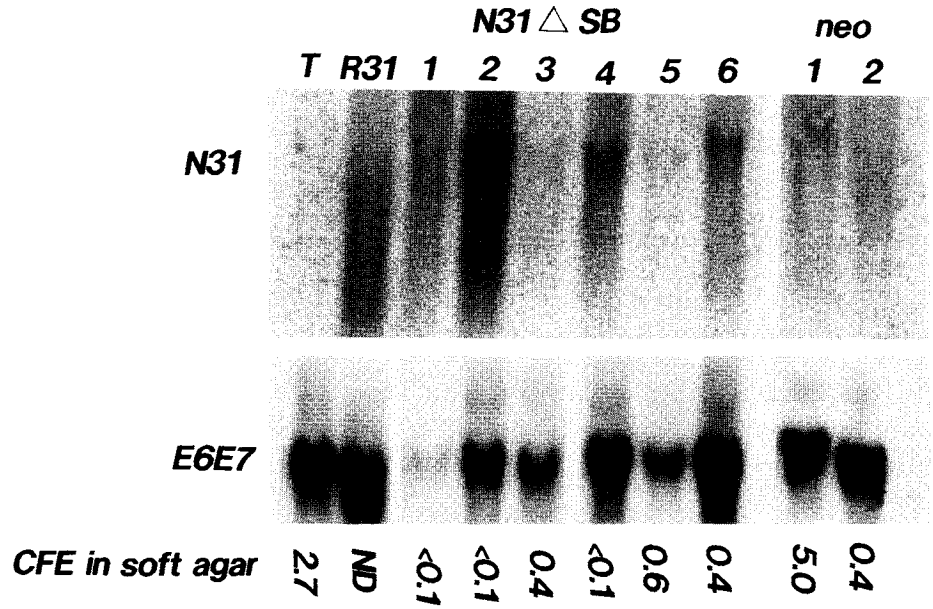

Fig. 5. Expression of N31 and soft agar assay in clones transfected with pSRαN31ΔSB-neo and pSRα-neo. Upper parts show the expression of N31 and lower parts show that of E6E7 gene. T and R31 indicate mRNA extracted from 18T3Y1 and R31 cells, respectively. Soft agar assay was done as described in materials and methods.

shown in Fig. 5, three of six clones transfected with pSRαN31ΔSB-neo had N31 expression at the same level as R31 (N31ΔSB, clones 2, 4 and 6). Of the three clones that overexpressed N31, two markedly reduced the colony-forming efficiency in soft agar (N31ΔSB, clones 2 and 4). N31ΔSB clone 1 did not form any colonies in soft agar, and this was thought to be due to the reduction of E6E7 mRNA rather than the expression of N31.

DISCUSSION

The region of HPV16 containing E6 and E7 is capable of transforming primary rat kidney cells in cooperation with exogenous activated ras (17). HPV16 DNA can also transform primary mouse kidney cells when co-transfected with activated ras or fos (6). We have also found that the HPV16 E7 gene immortalizes rat embryo fibroblast (REF), but that the E6 and E7 genes cannot transform these cells (12). After long passage of time, spontaneously transformed cells appear in E6 and E7 immortalized REF cells. The level of endogenous k-ras gene mRNA expressed in transformed cells is higher than that in immortalized cells. There are reports that chromosome 11 has genes that suppress tumorigenicity in HeLa cells (21) and that the incidence of loss of heterozygosity of the short arm of chromosome 3 is high in cervical cancer (28). Thus, the HPV E6 and E7 oncogenes alone are not sufficient for transformation of primary rodent cells or for cervical carcinogenesis. It is probable that additional genetic changes such as activation of proto-oncogenes or inactivation of tumor suppressor genes are required for carcinogenesis.

Using the somatic cell hybrid method, we previously found that REF may have genes that suppress transformation by the E6 and E7 genes (18). To obtain these genes, we transfected a REF cDNA expression library into E6E7 transformed cells and isolated 10 independent revertants. Although these revertants expressed E6 and E7 mRNAs at the same levels as the parent cells, they showed a satble non-transformed phenotype. In cell hybrids between revertants and 18T3Y1 cells, the non-transformed phenotype was dominant, as shown by soft agar assay. These findings indicated that revertants have a transformation-suppressive function, which might be due to expression of the transfected cDNA. However, we could not exclude the possibility that reversion was a dominant cellular process occurring spontaneously or was induced by insertion of the plasmid into the host gene. Therefore, it was necessary to recover the cDNA plasmids and examine their suppressive function.

It is difficult to recover integrated plasmids from cells transfected stably with a cDNA expression library. Eiden *et al.* (8) constructed a genomic DNA phage library of candidate cells and searched it using a cDNA library vector as a probe to recover transfected cDNA; however, this method seems troublesome. Cutler *et al.* (7) isolated a cDNA plasmid from Hirt supernatants of candidate cells fused with COS cells as the cDNA expression vector has an SV40 replication origin; however,

this method seems to be unstable. We tried to recover cDNA plasmids using the method previously reported by Noda *et al.* (20). This method is convenient, but the plasmids can only be recovered when they are integrated into the host gene as tandem repeats, and we obtained only three plasmids from two of ten revertants. Therefore, we modified this method to recover cDNA plasmids more efficiently. Plasmids were recovered from three revertants from which none was obtained by previous method. No cDNA plasmid could be recovered from five revertant cells. This may be because the pBR322 replication origin or ampicillin-resistance gene is not intact or the size of the fragment obtained with digestion does not permit plasmid replication. When the inserted cDNA has recognition sites for enzymes, the plasmids recovered will not have intact cDNA, and several recovered may have broken cDNA. The method must be improved to recover transfected cDNA in an intact form more easily and efficiently.

We found that the cDNA recovered from R31 was lost in re-transformed hybrid cells. This finding indicates that loss of the transfected cDNA induces re-transformation and that this cDNA may have a suppressive function on the transformation by the E6 and E7 oncogenes. We screened the REF cDNA library and obtained a pcD2 plasmid containing about 1.5 kb cDNA insert. As 1.0 kb and 1.7 kb mRNAs were detected in REF by Northern blot analysis, N31 insert cDNA was thought to be close to full length (19). Since N31 cDNA contained multiple translation terminal codons in all three frames, the encoded protein was not apparent. However, it has the mRNA degradation motif in the 3′ region and by deletion of the 3′ region containing this motif and use of SRα promoter, expression of N31 cDNA can be enhanced markedly. The 18T3Y1 cell clones that overexpress this cDNA reduced the colony-forming efficiency in soft agar. Recently, it was reported that the H19 gene, which has been suggested not to encode protein and to function as an RNA component (2), has tumor-suppressive activity (10). We are now investigating whether N31 encodes protein or function as RNA. Moreover, we found that one of cDNAs recovered from R56 weakly suppresses the colony formation in soft agar and tumorigenicity in nude mouse (13).

Acknowledgments. We thank Dr. P.M. Howley and Dr. K. Arai for providing p1571 and pcDLSRα296. This work was supported by grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

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(Received for publication, November 22, 1993
and in revised form, December 17, 1993)