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Neoplastic Transformation of Rat 3Y1 Cells by a Transcriptionally Activated Human c-*myc* Gene and Stabilization of p53 Cellular Tumor Antigen in the Transformed Cells

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Transformed foci were obtained in rat 3Y1 fibroblasts cotransfected wtih pR*myc* 27 (transcriptionally activated c-*myc*) and pSV2*neo* DNA. R*myc*Y cell lines (1 to 7) were established from these foci. R*myc*Y cells were small and round and contained enlarged nucleoli in the nucleus. The *myc* gene was expressed in these cell lines at a much higher level than in 3Y1 cells and at a level similar to that in HL-60 cells. These cell lines formed colonies in soft-agar culture and tumors in syngeneic rats transplanted with R*myc*Y cells. Expression of the gene and colony formation in soft-agar culture were analyzed in subclones from R*myc*Y cell line 1. A correlation between *myc* gene expression and the ability to form colonies in soft-agar culture was observed in these cells. Antibody against p53 cellular tumor antigen was detected in some sera from tumor-bearing rats. p53 cellular tumor antigen stabilized and accumulated in R*myc*Y cells to the same extent as in simian virus 40-transformed cells. The results suggest that elevated c-*myc* expression and an increased amount of p53 cause 3Y1 cells to become a more tumorigenic cell line.

The rearranged or amplified form of the c-*myc* oncogene has been found in a wide range of tumors (2–7, 12, 23, 34, 38, 42, 43, 45, 46), and the c-*myc* gene has been cloned from various human cancers (3, 5, 7, 28, 43; M. Shibuya and S. Yamaguchi, *in 5th International Workshop on Immune Deficient Animals*, in press). The activation of the c-*myc* gene appears to be clearly related to tumor formation. However, the causative significance of c-*myc* activation in tumorigenicity and the functions of the gene product have not yet been defined. The product of the *myc* gene has been reported to be localized in the nucleus (1, 8, 15, 26) and associated with the nuclear matrix (9). v-*myc* and c-*myc* gene products were shown to have a DNA-binding capacity in vitro (8, 26, 49).

Land et al. (22) reported that cotransfection of viral promotor-linked c-*myc* DNA and activated *ras* DNA transformed secondary rat embryo fibroblasts (REF). Since transfection of the activated *ras* gene alone, which carries a strong transforming activity in NIH 3T3 cells, did not transform REF, the viral promoter-linked c-*myc* gene may have a transformation helper function. Recently, it has been reported that immortalized rat fibroblast cell lines expressing a high level of c-*myc* oncogene were weakly tumorigenic in nude mice and syngeneic rats (18). More recently, Kelekar and Cole (19) reported that normal human and mouse c-*myc* genes driven by strong promoters are capable of inducing in mouse NIH 3T3 fibroblasts many properties of transformed cells without a greatly altered morphology. Furthermore, it is well known that in a chicken system, the v-*myc* gene alone is able to transform fibroblasts as well as monocytes and that the c-*myc* gene with the inserted viral promoter appears to be directly responsible for formation of B-cell lymphoma in the bursa Fabricius (16). These results indicate that transfection of the transcriptionally activated c-*myc* gene could lead to neoplastic transformation of cells under appropriate conditions in mammalian systems.

An immortalized rat cell line, 3Y1, has been successfully used for cell transformation by transfection with viral genes, including the adenovirus type 12 (Ad12) E1A gene (39). The Ad12 E1A gene has an immortalizing function similar to that of the c-*myc* gene (29, 33).

In this paper, we describe the transfection of rat 3Y1 cells with the activated c-*myc* gene. Transformed foci were formed and transformed cell lines were established. These cells formed colonies in soft agar and tumors in syngeneic rats. The c-*myc* gene and the p53 cellular tumor antigen were expressed at an increased level in these cells. A correlation was found between increased expression of the c-*myc* gene and the phenotype of the transformed cells. The results indicate that an elevated expression of c-*myc* gene and an increased amount of p53 appear to be involved in transformation of 3Y1 cells with the activated c-*myc* gene.

MATERIALS AND METHODS

Cells. Rat 3Y1 cells established from a Fisher rat embryo (20) were used for cell transformation. W3Y and Tu-5 cells were 3Y1 and mouse cells transformed with simian virus 40 (SV40) (37, 51). REF 10-1 was a secondary REF line transformed with long terminal repeat (LTR)-*myc* and *ras* DNA (Shibuya and Yamaguchi, in press). E1AY cells were 3Y1 cells transformed with the Ad12 E1A gene (K. Shiroki, unpublished data). HL-60 is a human promyelocytic leukemic cell line (7). These cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Plasmid DNA. pR*myc* 27 refers to pBR322 into which was inserted the c-*myc* DNA (exons 2 and 3) and two copies of the Rous sarcoma virus LTR between the *Eco*RI and *Pvu*II sites of the vector (Fig. 1) (Shibuya and Yamaguchhi, in press). By nucleotide sequence analysis, the predicted amino acid sequence of this human c-*myc* gene was shown to be essentially the same as that of the gene from normal placenta, although one silent base change was found in the coding region of exon 2 (48, 50). pSV2*neo* (44) was used for selection of transfected cells.

Transformation of 3Y1 cells. Cotransfection of circular forms of pR*myc* 27 (10 μg per dish) and pSV2*neo* (2 μg per

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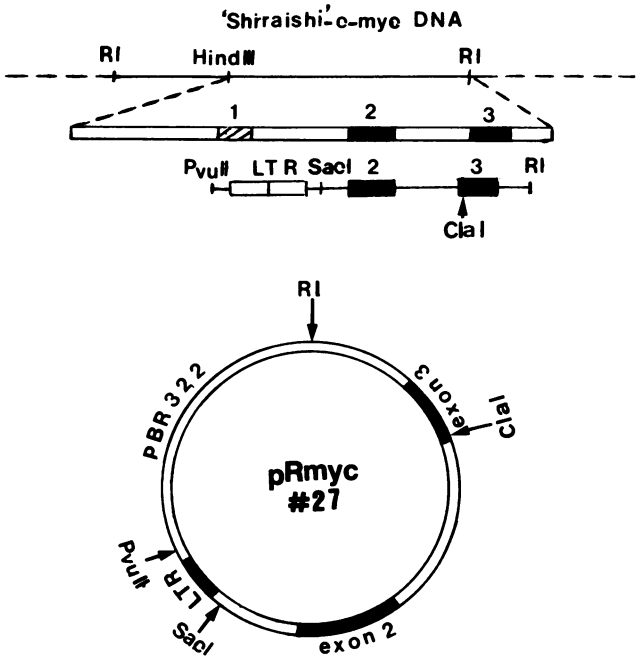

FIG. 1. Structures of recombinant plasmids used. Upper part shows the structure of the molecularly cloned Shiraishi c-*myc* DNA (*Hin*dIII-*Eco*RI, 8 kb). The *Sac*I-*Eco*RI 4-kb Shiraishi c-*myc* DNA (partial digestion at the 3' *Sac*I site in intron 1) which contains exons 2 and 3 (solid box) was ligated to the *Pvu*II-*Sac*I 1.0-kb fragment of Rous sarcoma virus LTR (open box). This ligated DNA fragment was cloned into pBR322 at *Eco*RI and *Pvu*II sites (pR*myc* 27) (Shibuya and Yamaguchi, in press).

dish) DNAs was carried out by the calcium phosphate technique described previously (13, 39, 40). About 40 h after transfection, the cells were trypsinized and replated at a 1:4 dilution. On the next day, G418 was added to the medium at a concentration of 200 μg/ml. The medium plus drug was changed every 4 days. The cultures were kept at 37°C until G418-resistant colonies appeared.

Preparation and blot analysis of mRNA. Cytoplasmic RNAs were extracted as described previously (35, 40). The RNA was denatured and electrophoresed on 1.0 to 1.4% agarose gels containing formaldehyde. After electrophoresis, the RNA was transferred to a nitrocellulose filter, immobilized, and hybridized with a nick-translated DNA probe (31). The *Eco*RI-*Cla*I fragment about 1,500 base pairs long from pR*myc* 27 DNA was used as a probe.

Colony formation in soft agar. Transformed cells were plated into soft-agar medium and kept at 37°C as described previously (39, 40). The formation of colonies was examined 4 weeks after plating.

Formation of tumors in rats. A suspension of transformed cells was transplanted into newborn Fisher rats (10^6 cells per rat) as described previously (39, 40). The formation of tumors was observed for 3 months.

Immunofluorescence. SV40-infected cells and R*myc*Y 1, REF 10-1, and Tu-5 cells grown on cover slips were rinsed, dried, and fixed with acetone. The cells were treated with serum from a rat bearing tumors, stained with anti-rat immunoglobulin G immunoglobulin conjugated with fluorescein isothiocyanate, and observed in a fluorescent microscopy (39, 40).

Analysis of p53 protein synthesis. Cells were grown in 60-mm dishes and labeled with 50 μCi of [^{35}S]methionine per dish for 10 h. Then the cells were washed with phosphate-buffered saline and extracted into 200 to 300 μl of lysis buffer (100 mM Tris hydrochloride [pH 8.0], 10 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 0.3 μg of phenylmethylsulfonyl fluoride per ml) at 0°C. The cell lysates were precleared by absorption onto *Staphylococcus aureus*. Equal amounts of radioactive protein were immunoprecipitated with either monoclonal antibody PAb122 to p53 (14) or monoclonal antibody 401 to Ad12 E4 protein (40). Antigen-antibody complexes were collected by centrifugation after adsorption onto *S. aureus*. The precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (21, 37, 41). Monoclonal antibody 401 was used as a control.

p53 complex formation. [^{35}S]methionine-labeled cellular extracts were prepared from Tu-5 and R*myc*Y 1 cells, applied to a 5 to 20% glycerol gradient in 100 mM Tris hydrocholride (pH 8)–100 mM NaCl–0.5% Nonidet P-40–0.3 μg of phenylmethylsulfonyl fluoride per ml and centrifuged for 17 h at 40,000 rpm (24, 47). After fractionation, a sample from each fraction was immunoprecipitated with monoclonal antibody PAb122 and analyzed by SDS-PAGE.

Stability of p53. Cells were labeled with 50 μCi of [^{35}S]methionine per dish for 2 h and chased with fresh medium containing 1 mg of cold methionine per ml. At 0, 3, 7, 16, and 28 h after labeling, cellular extracts were prepared, immunoprecipitated with monoclonal antibodies, and analyzed by SDS-PAGE.

RESULTS

Establishment and characterization of rat 3Y1 cells transformed with activated c-*myc* DNA. Rat 3Y1 cells were co-transfected with pR*myc* 27 and pSV2*neo* DNA (Fig. 1) in a 5:1 ratio and cultured in G418 selection medium. At 3 weeks after transfection, dense transformed colonies were observed in a background of flat G418-resistant colonies (Fig. 2A). Approximately seven dense colonies were observed in 10^6 transfected 3Y1 cells. Some of the flat colonies consisted of normal 3Y1 cells and morphologically transformed 3Y1 cells. In the cell lines from later colonies, the *myc* gene was expressed at low levels (data not shown). Only flat colonies of normal 3Y1 cells were detected with 3Y1 cells transfected with pSV2*neo* DNA alone. A dense colony consisted of multilayered transformed cells, which made it difficult to differentiate each cell type (Fig. 2A). Transformed cell lines were established from the dense foci and named R*myc*Y 1 to 7. R*myc*Y cells were round and small and contained enlarged nucleoli in the nuclei (Fig. 2B) compared with untransformed 3Y1 cells (Fig. 2C).

In soft-agar culture, R*myc*Y cell lines formed colonies efficiently (Table 1). When these cell lines were transplanted subcutaneously into newborn rats, tumors were formed with the same efficiency and latency as with Ad12-transformed cells (Table 2) The presence of antibody to the c-*myc* gene product in sera from tumor-bearing rats was examined by immunofluorescence in R*myc*Y 1 and REF 10-1 cells, in which the c-*myc* gene was expressed at a high level (Shibuya and Yamaguchi, in press). All sera tested gave negative results, indicating that no antibody to the c-*myc* gene product was detected. When these sera were examined in SV40-transformed W3Y and Tu-5 cells, examined in the same manner, some gave positive results (Fig. 3). When these positive sera were used for immunoprecipitation of extracts from ^{35}S-labeled Tu-5 cells, a band analogous to p53 cellular tumor antigen was detected (Fig. 4, lane 1). The result

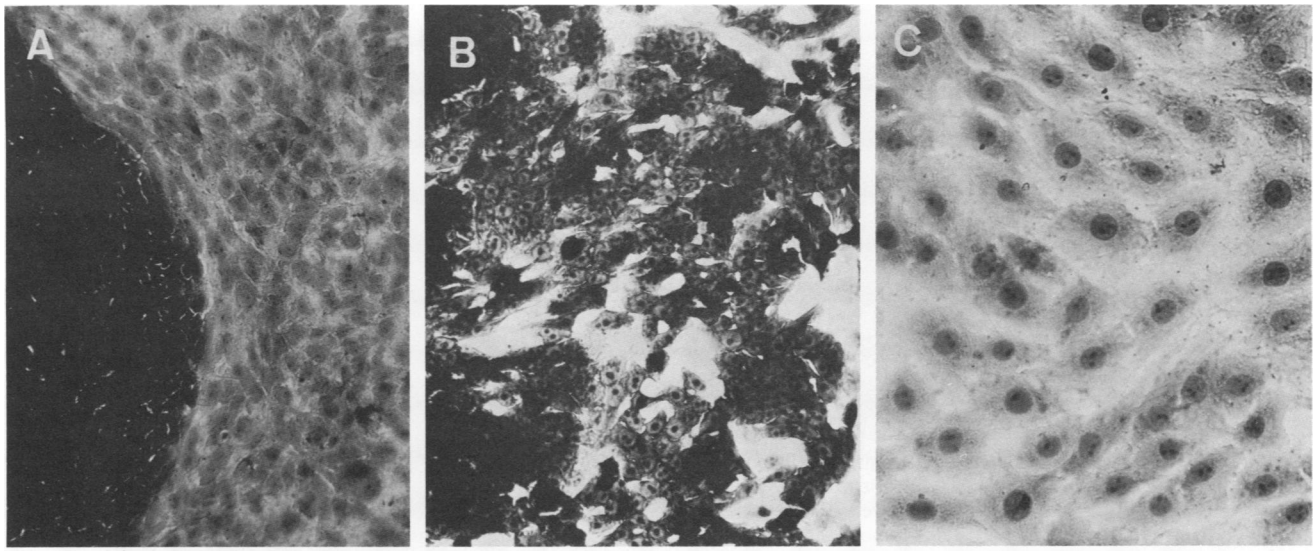

FIG. 2. Part of a transformed dense colony of 3Y1 cells transfected with pR*myc* 27 and pSV2*neo* (A). Morphology of R*myc*Y 1 (B) and untransformed 3Y1 (C) cells. Panels B and C have the same enlargement.

indicates the presence of antibody to p53 in these positive sera and suggests that there is an increased amount of p53 in these transformed cells. The suggestion was confirmed by immunoprecipitation of p53 with monoclonal antibody PAb122 to p53 (Fig. 4, lane 2).

Cytoplasmic RNAs from R*myc*Y cells were examined by Northern blot analysis with ^{32}P-labeled *myc* DNA as a probe (Fig. 5). Transcripts from pR*myc* 27 DNA were detected in R*myc*Y 1 to 4 cells at a substantially higher level than that in HL-60 cells. The sizes of the transcripts in R*myc*Y cells were, however, different from that in HL-60 cells: HL-60 contained a normal-sized, 2.3-kilobase (kb) c-*myc* mRNA, whereas R*myc*Y cells showed a major species of exogenous c-*myc* RNA of approximately 2.1 kb and a minor species of 3.5 kb. The size of the 2.1-kb RNA is consistent with that expected from the structure of LTR–c-*myc* DNA. Therefore, we consider that the 2.1-kb c-*myc* RNA indicates the mature mRNA transcribed from the pR*myc* 27 DNA transfected into 3Y1 host cells. The minor species, the 3.5-kb c-*myc* RNA, possibly is an immature precursor mRNA without splicing of intron 2, although this possibility was not confirmed. Transcripts of other c-*onc* genes, H-*ras* and K-*ras*, were not detected in R*myc*Y cells (data not shown).

Correlation between *myc* expression and soft-agar colony-forming abilities of subclones derived from R*myc*Y 1 cells. When R*myc*Y 1 cells were cultured at reduced cell numbers (100 to 1 cells per well) in 48 tissue culture wells, colonies of various phenotypes (dense or flat) were observed. Subclones were established from those colonies. When these subclones were cultured in soft agar, they showed variable efficiency for colony formation (Table 1). One of the subclones, R-q, formed colonies at the same efficiency as the original R*myc*Y 1 cells. Some subclones (R-d, R-3, R-h, and R-i) formed colonies at a slightly reduced efficiency from that of R*myc*Y

TABLE 1. Colony formation of R*myc*Y 1 cells and subclones in soft-agar culture

Transformed cell clones	No. of colonies per plate with the following no. of cells/plate[a]		
	10^4	10^3	10^2
R*myc*Y 1	>200, L	>200, L	>50, L
R-a	>50, S	≧10, S	0
R-b	≧10, S	0	0
R-c	0	0	0
R-d	>200, L, S	≧10, M	0
R-e	>200, L	>50, L	≧10, M
R-f	≧10, S	0	0
R-g	≧10, S	10, S	0
R-h	>200, S	>50, S	≧10, S
R-i	>200, M	>50, M, L	≧50, M, L
R-j	0	0	0
R-k	>50, M	>50, M	≧10, M
R-l	>50, M	>50, M	≧10, M
R-m	0	0	0
R-n	10, S	<10, S	0
R-o	0	0	0
R-p	0	0	0
R-q	>200, L	>200, L	>50, L

[a] The transformed cells were seeded at 10^2, 10^3, and 10^4 cells per 5-cm dish. The numbers of colonies per dish 4 weeks after seeding are shown by numerals. 0, No colony formed. Sizes of colonies are shown as follows: L, 2 mm; M, 1 to 2 mm; S, 1 mm in diameter.

TABLE 2. Tumor formation in rats transplanted with transformed cell lines[a]

Transformed cell lines	Tumor formation at:	
	60 days	90 days
R*myc*Y		
1	6/8	8/8
1	4/6	6/6
2	3/5	5/5
Ad12-transformed cells[b]		
12WY 1-1	5/6	6/6
HY1	0/7	3/7
Untransformed 3Y1 cells	0/6	0/6

[a] Transformed cells were transplanted subcutaneously into newborn Fisher rats at 24 h after birth at 2×10^6 cells per rat. Results are shown as number of tumor-bearing rats/number of rats tested.

[b] 12WY 1-1 cells were 3Y1 cells transformed with Ad12. HY1 cells (41) were 3Y1 cells transformed with 0 to 4.6 map units of Ad12 DNA.

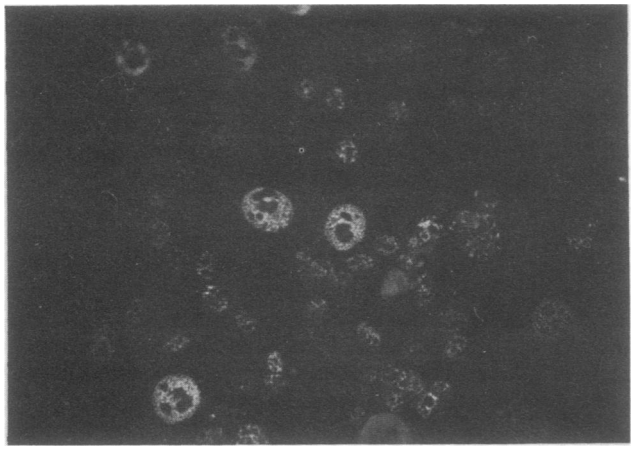

FIG. 3. Immunofluorescent detection of antibody to the p53 cellular tumor antigen in a serum from a tumor-bearing rat. Acetone-fixed Tu-5 cells (SV40-transformed cells) on cover slips were treated with serum from a rat bearing R*myc*Y 1 cell tumors and stained with fluorescent dye-conjugated rabbit immunoglobulin against rat immunoglobulin.

1 cells. Other subclones (R-c, R-j, R-m, and R-p) formed no colonies (Table 1).

Expression of the *myc* gene in these subclones was examined by the Northern blot method with ^{32}P-labeled *myc* DNA as a probe (Fig. 6). In the R-q cell clone, expression of the c-*myc* gene was at the same level as in R*myc*Y 1 cells. In the R-d, R-e, R-h, and R-i cell clones, the *myc* mRNA was detected only at reduced levels. In other subclones (R-c, R-j, R-m, and R-p) which had no colony-forming activity in soft agar, expression of the exogenously introduced c-*myc* gene was greatly reduced. Thus, a good correlation was found between colony-forming abilities in soft agar and the levels of expression of c-*myc* DNA among these subclones.

Characterization of p53 cellular tumor antigen in R*myc*Y cells. Since antibodies to p53 were detected in some sera

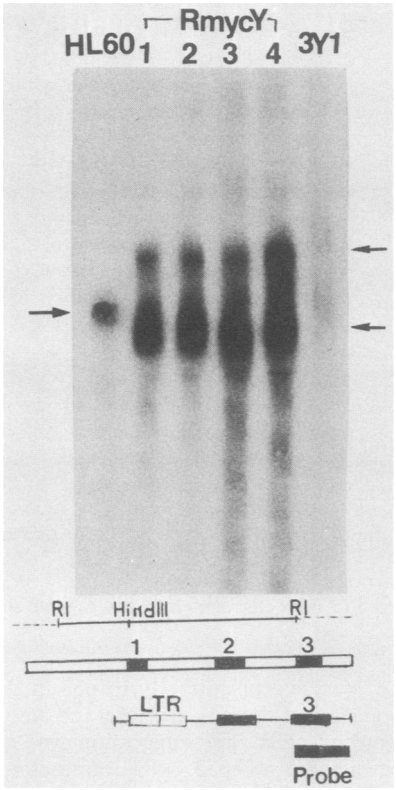

FIG. 5. Expression of transfected c-*myc* gene in R*myc*Y cells. Cytoplasmic RNA (10 μg) from 3Y1, R*myc*Y 1, R*myc*Y 2, R*myc*Y 3, and R*myc*Y 4 cells and cytoplasmic RNA (4 μg) from HL-60 cells were analyzed by Northern blotting with the human c-*myc* probe shown under the gel. Arrow at the left side of the gel indicates 2.3-kb endogenous c-*myc* mRNA. Arrows at the right side of the gel represent 3.5- and 2.1-kb exogenous c-*myc* mRNA.

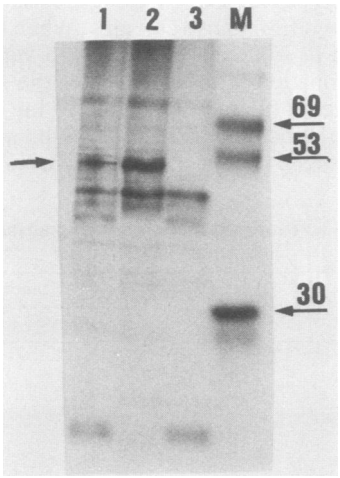

FIG. 4. Immunoprecipitation of the p53 cellular tumor antigen with serum from a tumor-bearing rat. The extract from ^{35}S-labeled Tu-5 cells was immunoprecipitated with serum from a tumor-bearing rat (lane 1), monoclonal antibody PAb122 to p53 (lane 2), and normal rat serum (lane 3). Immunoprecipitates were analyzed by 10% PAGE. Lane M, ^{14}C-labeled molecular weight markers; phosphorylase *b* (97,000), bovine serum albumin (69,000), gamma globulin heavy chain (53,000), and carbonic anhydrase (30,000). Arrow indicates p53 cellular tumor antigen.

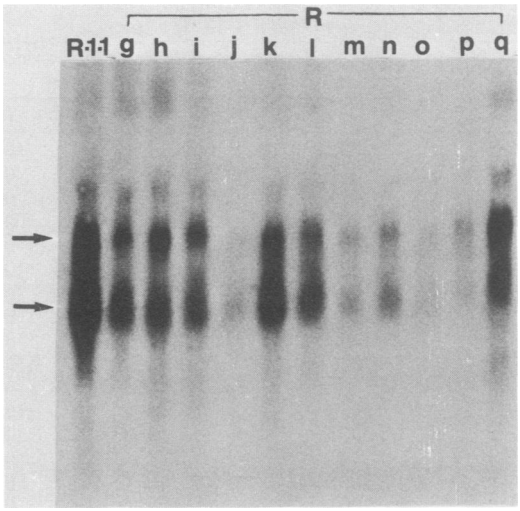

FIG. 6. Expression of the transfected c-*myc* gene in subclones from R*myc*Y 1 cells. Cytoplasmic RNA (10 μg) from R*myc*Y 1 and its subclones R-g, R-h, R-i, R-j, R-k, R-l, R-m, R-n, R-o, R-p, and R-q was analyzed by Northern blotting with the human c-*myc* probe indicated in Fig. 5. Arrows indicate 3.5-kb (top) and 2.1-kb (bottom) exogenous c-*myc* mRNA.

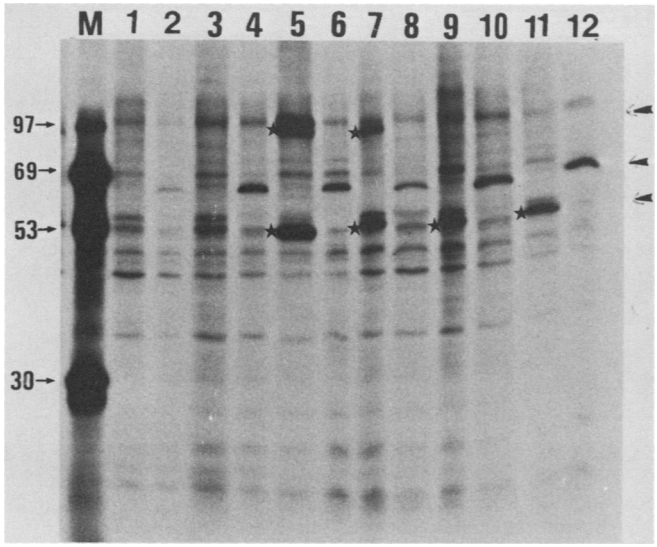

FIG. 7. Immunoprecipitation of p53 cellular tumor antigen. Extracts of ^{35}S-labeled 3Y1 (lanes 1 and 2), E1AY (lanes 3 and 4), Tu-5 (lanes 5 and 6), W3Y (lanes 7 and 8), R*myc*Y 1 (lanes 9 and 10), and R-q (lanes 11 and 12) cells were immunoprecipitated with PAb122, a monoclonal antibody to p53 (lanes 1, 3, 5, 7, 9, and 11). Monoclonal antibody 401 to Ad12 E4 protein (lanes 2, 4, 6, 8, 10, and 12) was used as a control. Immunoprecipitates were analyzed by 10% PAGE. Lane M, ^{14}C-labeled molecular weight markers as described in the legend to Fig. 4. ★, SV40 T antigen (upper) and p53 (lower).

from rats bearing R*myc*Y 1 cell tumors, the amount and stability of the p53 cellular tumor antigen was further studied by in vivo labeling and immunoprecipitation of R*myc*Y 1 cells. Cell extracts labeled with [^{35}S]methionine were immunoprecipitated with monoclonal antibody PAb122 to p53 and analyzed in SDS-polyacrylamide gels (Fig. 7). The amount of p53 detected in R*myc*Y 1 and R-q cells increased significantly and almost at the same level as in SV40-transformed Tu-5 and W3Y cells. In Tu-5 and W3Y cells, SV40 large T antigen was coprecipitated with p53 (Fig. 7, lanes 5 and 7).

We then examined the native state of p53 cellular tumor antigen in R*myc*Y 1, Tu-5, and 3Y1 cells by glycerol gradient centrifugation (Fig. 8). In 3Y1 cells, most of the p53 was detected in a free form in the upper part of the gradient (Fig. 8C). In Tu-5 cells, most of the p53 was detected in a form complexed with SV40 large T antigen in the lower part of the gradient (Fig. 8B). In R*myc*Y cells, p53 was detected in many fractions covering the middle and lower parts of the gradient (Fig. 8A). The result suggests that p53 is present in a form complexed with a factor(s) in R*myc*Y cells. However, the suggestion could not be confirmed, because neither the nature of the complexed factor nor the reason for the broad distribution of p53 could be clarified (Fig. 8A).

The stability of p53 in R*myc*Y, Tu-5, and 3Y1 cells was examined by pulse-chase experiments (Fig. 9). Immediately after labeling, the intensity of the p53 band was at almost the same level among 3Y1, Tu-5, and R*myc*Y cells. At 7 h after pulse-labeling, p53 was detected at a reduced level in 3Y1 cells owing to the normal turnover, but was found at a high level in Tu-5 and R*myc*Y cells. At 28 h after pulse-labeling, p53 was still clearly recognized in Tu-5 and R*myc*Y cells. The result shows that p53 is present in a stable form in R*myc*Y cells.

These results indicate that in R*myc*Y cells, p53 is present in an increased amount, possibly owing to its increased

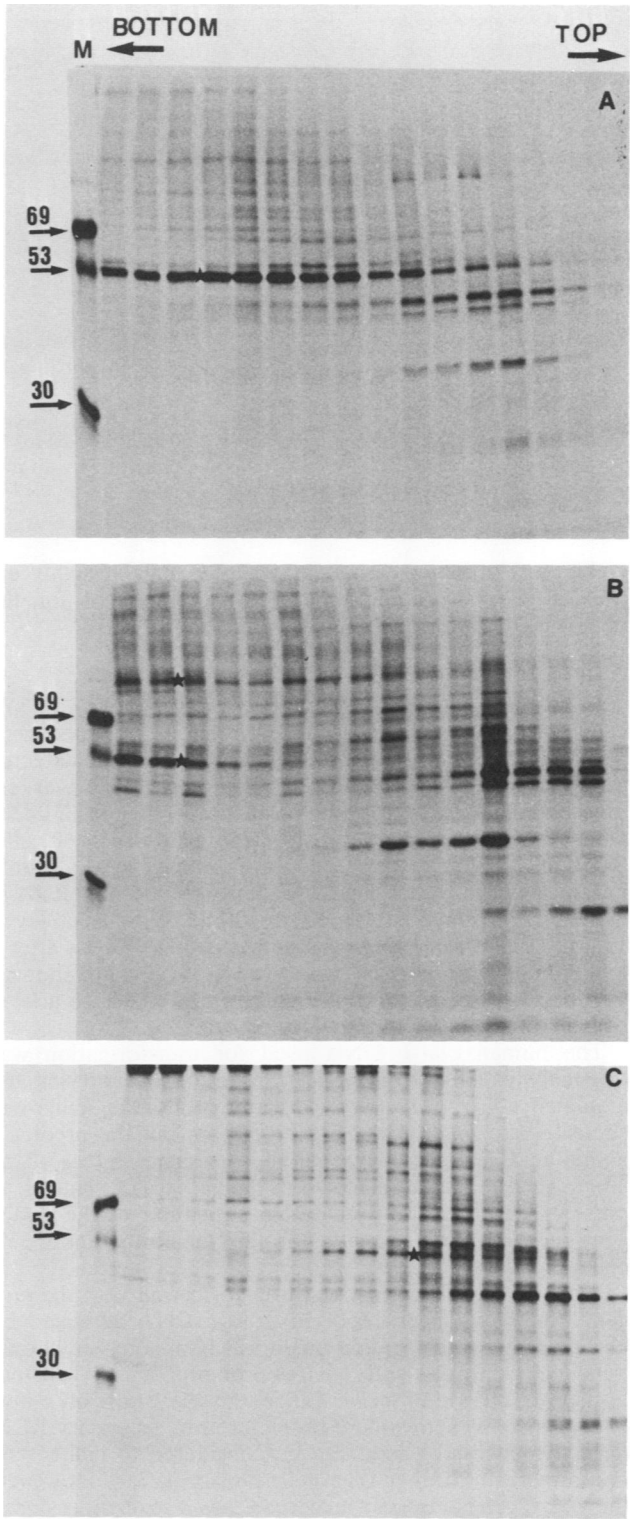

FIG. 8. Sedimentation analysis of p53. Cell extracts from ^{35}S-labeled R*myc*Y 1 (A), Tu-5 (B), and 3Y1 (C) cells were centrifuged through 5 to 20% linear glycerol gradients. A sample from each fraction was immunoprecipitated with 20 μl of monoclonal antibody to p53 and analyzed by SDS-PAGE. Lane M ^{14}C-labeled molecular weight markers as described in the legend to Fig. 4. ★, SV40 T antigen (B) and p53 (A, B, and C).

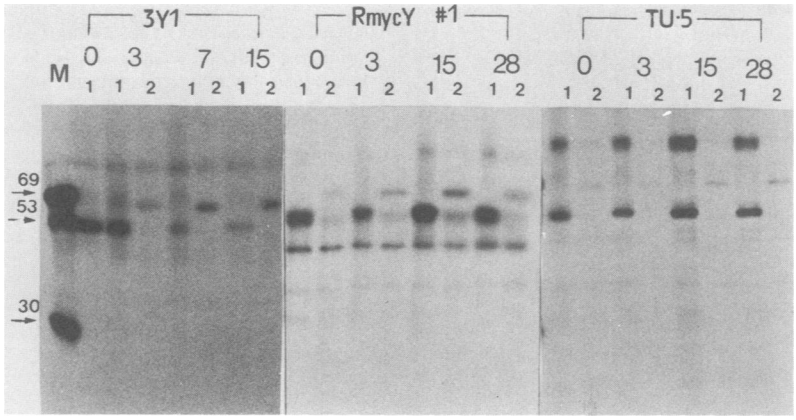

FIG. 9. Turnover of p53 cellular tumor antigen in R*myc*Y 1 cells. 3Y1, R*myc*Y 1, and Tu-5 cells were labeled with [^{35}S]methionine for 2 h. Cell extracts were prepared immediately and after chases for 3, 7, 15, and 28 h as indicated under the name of the cells. A sample (5×10^6 cpm) from each cell extract was immunoprecipitated with monoclonal antibody PAb122 (lane 1) or monoclonal antibody 401 (lane 2). Precipitates were analyzed by SDS-PAGE. Lane M, ^{14}C-labeled molecular weight markers as described in the legend to Fig. 4.

stability. The increased amount of p53 and the highly expressed c-*myc* protein appear to be important for the neoplastic transformed phenotype of R*myc*Y cells.

DISCUSSION

In this study, we demonstrated transformation of rat 3Y1 cells cotransfected with activated human c-*myc* oncogene and pSV2*neo*. Drastic morphological changes were detected in the c-*myc*-transformed cells. The c-*myc* gene was expressed in these cells at a much higher level than in 3Y1 cells and at a similar or slightly higher level than in HL-60 cells. Since the amplification of endogenous *myc* gene was 20-fold in HL-60 cells (7), the degree of elevation of c-*myc* RNA in R*myc*Y cells appears to be 20- to 40-fold of the basal level. Endogenous c-*myc* RNA was detected in 3Y1 cells after a longer exposure than that shown in Fig. 5 (data not shown). To our surprise, p53 was elevated in these cells to a level similar to that in SV40-transformed cells.

The human c-*myc* DNA used for transformation was originally isolated from a gastric carcinoma carrying the amplified c-*myc* gene (38). This c-*myc* DNA contains one silent base change in the coding region, but the predicted amino acid sequence is essentially the same as that of the c-*myc* gene in human placenta (Shibuya and Yamaguchi, in press). Therefore, the qualitative activation of the c-*myc* gene seems not to be involved in the transformation of 3Y1 cells.

In mammalian systems, cell transformation with the *myc* gene has rarely been reported; c-*myc* DNA activated by chromosomal translocation in Burkitt lymphoma or normal c-*myc* DNA linked with a viral promotor usually did not transform NIH 3T3 cells. Since the elevation of c-*myc* expression was shown here to immortalize secondary REF, an activated c-*myc* gene can be considered to induce cell immortalization rather than cell transformation. However, the v-*myc* gene of avian retroviruses has a strong transforming activity on chicken fibroblasts and bone marrow cells, and an elevated c-*myc* expression has recently been demonstrated to induce cell transformation in murine cells (18, 19). In the latter case, the increase of the c-*myc* mRNA was reported to be two- to sixfold. From these results and our data, we suggest that the quantitative activation of the c-*myc* gene can induce cell transformation itself under some appropriate conditions.

When 3Y1 cells were transfected with the Rous sarcoma virus LTR-linked human c-*myc* gene, pR*myc* 27, a few dense foci were formed. In most of these experiments, many flat foci were formed after G418 selection, and cells in some of the flat foci showed slight changes in morphology. In these cells, the human c-*myc* gene was expressed at a reduced level. Although it is not clear why dense foci in our system were formed at a low efficiency, there are several possible explanations for this result. (i) Since we found a very high level of c-*myc* mRNA in transformed cells and showed a clear correlation between c-*myc* expression and morphological changes in transformed cells, it seems likely that high expression of the c-*myc* gene is necessary to transform 3Y1 cells. c-*myc*-transfected 3Y1 cells with a low expression of the c-*myc* gene do not show the transformed phenotype, simply owing to insufficient c-*myc* expression for morphological change. (ii) Alternatively, a high level of c-*myc* mRNA in 3Y1 cells is not enough for full transformation, and activation of another cellular factor or factors is required for the transforming ability. In 3Y1 cells, stabilization and accumulation of p53 cellular tumor antigen might be the second factor for establishment of transformation.

The product of the c-*myc* gene and p53 are thought to provide an immortalizing function (11, 17, 22, 25, 30). The product of the *myc* gene has been reported to be localized in the nucleus (1, 8, 15, 26), to associate with the nuclear matrix (9), and to have a DNA-binding capacity (8, 26, 50). p53 has also been reported to be localized in the nucleus of transformed cells and in the cytoplasm of untransformed cells (32). p53 is generally unstable in untransformed cells but stable in transformed cells (32). Both p53 and the product of the c-*myc* gene are phosphorylated (15, 17). In mitogenic stimuli, the c-*myc* gene is inducible in the early G1 phase (32), and p53 is inducible in the middle to late G1 phase (30, 32). Although the function of both *myc* and p53 cellular tumor antigen are unclear, they may be different and may complement each other in cell transformation in some cell types such as 3Y1 cells.

It is well known that in virus-transformed cells, p53 binds to DNA tumor virus antigens such as SV40 large T antigen (24) and adenovirus-encoded E1B-58,000-dalton protein (36). In Meth A cells, chemically transformed mouse fibroblasts, p53 binds a cellular 68,000-dalton protein (10). Recently, Pinhasi-Kimhi et al. (27) reported that p53 formed

specific complexes with major heat shock proteins (68,000 to 70,000 daltons) in some transformed rat cells with activated H-*ras* and a p53-specific plasmid. Our next project may be to study whether p53 is complexed with a factor(s) in R*myc*Y cells.

The good correlation between *myc* gene expression and the ability to form colonies in soft-agar culture was observed in subclones from R*myc*Y 1 cells. However, a correlation between the stability of p53 and the ability to form colonies in soft-agar culture has not yet been confirmed. p53 was detected at a higher level in R-p clones than in R*myc*Y 1 cells (data not shown). Thus, a study of the functions of the c-*myc* gene and p53 is necessary to understand the mechanism of cell transformation.

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