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BIOCHEMICAL CORRELATES OF PHENOTYPIC REVERSION IN INTERFERON-TREATED MOUSE CELLS TRANSFORMED BY A HUMAN ONCOGENE

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Mouse interferon (IFN) induced a phenotypic reversion in RS 485, a clonal line of NIH 3T3 oncogenically transformed by a human c-Ha-ras1 gene activated by Ha-MuSV long terminal repeats (LTRs). Transfected c-Ha-ras DNA, unchanged in quantity and distribution, as compared to the parental RS 485 transformed cells, was still present in these revertants; however, there was a significant reduction in the amount of c-Ha-ras specific mRNA and of c-Ha-ras specified p21 protein.

Oncogenesis in some cases appears to involve activation of cellular transforming genes (c-oncs). Harvey murine sarcoma virus (Ha-MuSV), is capable of transforming fibroblasts in tissue culture (1). The transforming gene of Ha-MuSV has been designated as v-Ha-ras; the human cellular homologue of v-Ha-ras, as human c-Ha-ras (2); the ras gene encodes for a protein of 21 kd (p21). The overproduction of p21 in cells transfected with LTR activated c-Ha-ras1 is associated with oncogenic transformation.

We recently reported on the effect of mouse L-cell IFN (200 $IUml^{-1}$) on RS 485, a clonal line of NIH 3T3 transformed by human c-Ha-ras1 gene activated by the Ha-MuSV LTR (2,3). These cells were carried in the presence of IFN for approximately 10 to 20 cell generations, when flat revertant colonies were identified in the IFN-treated cultures seeded at low cell density; these colonies were isolated and cloned for further study. We observed no revertant colonies in untreated RS 485 cultures. Parental transformed RS 485 cells were refractile, fibroblast-like with cellular overlapping; they reached high saturation densities, formed colonies in soft agar, and tumors in nude mice.

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The isolated flat revertant clonal lines, however, consisted of well-spread epithelial-like cells, which were contact inhibited, had a low saturation density, and did not grow in soft agar; therefore, they exhibited a normal NIH 3T3 phenotype with respect to cell growth and morphology (3). Like NIH 3T3, revertant RS 485 cells failed to produce tumors in nude mice (manuscript in preparation).

The emergence of revertants in RS 485 treated with IFN was not due to a selective effect of IFN on the growth of transformed cells because: (i) the RS 485 lines employed in these studies were cloned from single cell cultures that were stable in that we observed no spontaneous revertants colonies in an extensive search of numerous passages; (ii) cessation of IFN treatment of RS 485 revertants resulted in appearance of retransformant, phenotypically oncogenic foci in the cultures (3); (iii) concentrations of IFN that had no significant effect on the growth of NIH 3T3 or RS 485 cells also induced morphologic reversion of RS 485; and, (iv) IFN-treated RS 485, that had not reverted, proliferated at a higher rate and reached a higher saturation density than did NIH 3T3 or RS 485 revertants, untreated or IFN-treated.

In the present study we report that in revertant RS 485 transfected c-Ha-ras DNA is still present, unchanged in genomic distribution and quantity as compared to the parental tumor cells, but there is a reduction in the amount of c-Ha-ras specific mRNA and of c-Ha-ras specified p21 protein.

MATERIALS AND METHODS: Preparation of high molecular weight DNA: High molecular weight DNA was prepared from confluent cell monolayers by the procedure of Gross-Bellard et al. (4) with slight modification; 3 to 5 x 10^7 cells were lysed with 14 ml of 10 mM Tris-HCl (pH 8), 10 mM EDTA, 10 mM NaCl and 0.5% SDS. The viscous lysate was digested overnight at 37°C with proteinase K (final concentration of 50 ug/ml). The lysate was extracted twice with phenol saturated with 0.1 M Tris-HCl (pH 9) and once with chloroform and the aqueous phase precipitated with 2.5 vol ethanol. After ethanol precipitation, the DNA was centrifuged and redissolved in 0.01 M NaCl, 0.05 M Tris (pH 8.0) and 10 mM EDTA. It was then treated with RNAse A and T_1 (Sigma) for 60 minutes at 37°C, phenol-extracted two more times and dialyzed at 4° for two days against 10 mM NaCl, 10 mM Tris (pH 8.0) and 0.5 mM EDTA.

Agarose gel electrophoresis of DNA and DNA transfer: Electrophoresis of high molecular weight DNA or restriction enzyme-treated DNA samples was carried out in a horizontal gel apparatus (Bethesda Research Labs) in 0.6% agarose gels containing 20 mM sodium acetate (NaOAc), 40 mM Tris-HCl (pH 8.1), 2 mM EDTA, and 0.5 ug/ml ethidium bromide; then, 20 ug of HMW DNA was incubated for 16

hours with EcoRI to allow complete digestion, and subjected to electrophoresis at 45 V for 30 hours at room temperature.

DNA to be hybridized with nick-translated, fragment-specific probes labelled with ^{32}P was transferred from agarose gels to nitrocellulose filters (Schleicher and Schuell) by the procedure of Southern with minor modifications (5). The gels were UV-irradiated for 15 minutes, then alkali treated with 0.3 N NaOH, 0.9 M NaCl for 45 minutes at room temperature. The gels were then neutralized with 1 M Tris-HCl (pH 7.5), 0.9 M NaCl and the DNA transferred from the gel to the filter by capillary action for at least 16 hours. The filters were then dried *in vacuo* at 80°C for two hours.

Isolation of mRNA from cultured cells: Cells were pelleted (1000 x g) after being washed in cold PBS. The cell pellet was resuspended in an equal volume of lysis buffer (10 mM Hepes pH 7.6, 1.5 mM MgAc, 90 mM KCl, 0.5% v/v NP40) vortexed, and centrifuged in a microfuge (4°C) for 10 minutes. The supernatants were then diluted seven-fold with buffer (50 mM NaAc pH 5, 10 mM EDTA, 0.5% SDS) on ice, and mixed with an equal volume of 1:1 Phenol/$CHCl_3$, then centrifuged at 15,000 rpm for 15 minutes at room temperature. The aqueous supernatants were re-extracted with phenol/$CHCl_3$, and once with one volume of chloroform. RNA was precipitated with two volumes of absolute ethanol overnight at -20°C. The bulk cytoplasmic RNA was pelleted by centrifugation at 15,000 rpm for 20 minutes, then dissolved in sterile H_2O, and its OD_{260} determined.

Hybridization reagents and conditions: For DNA:DNA or RNA:DNA hybridization studies, the probe used was gel-purified DNA fragments labelled with ^{32}P *in vitro* prepared by the repair synthesis ("nick-translation") reaction of *Escherichia coli* DNA polymerase (6). The reaction used 100 uCi each of [α-^{32}P]dCTP and [α-^{32}P]TTP (New England Nuclear Corp., 300 Ci/mmol); the specific activity of the double-stranded [^{32}P] human c-Ha-*ras*1 DNA probes was 10^8 cpm/ug. Nitrocellulose blots were presoaked at 40°C in the hybridization mix for two hours. The hybridization mix contained 40% formamide, 10% dextran sulfate, 5 x SSC, 4 x Denhardt's reagent (0.02% wt/vol each of BSA, PVP, and Ficoll), 10 mM Tris-HCl pH 7.5, and 250-500 ug/ml sonicated, denatured salmon testis DNA; 1.5 x 10^6 cpm/ml of ^{32}P-nick-translated DNA probe, heated and denatured (100°C/10-15 minutes), was added to the hybridization mix. After 16-20 hours incubaton at 40°C, the filters were washed with 2 x SSC, 0.1% SDS for 3 x 20 minutes at room temperature and then with 0.1 x SSC, 0.1% SDS for 2 x 20 minutes at 50°C. The filters were then dried and exposed at -70°C to Kodak x-ray film, with a DuPont Lightning Plus intensifying screen (7).

Dot blot hybridization of cytoplasmic RNA: A nitrocellulose filter was equilibrated in 15 x SSC; 10 ug of RNA was serially diluted 1:2 in 15 x SSC and spotted onto the filter. After baking the blot at 80°C for two hours, hybridization was performed as described above with either c-Ha-*ras* specific, actin-specific, or type I collagen-specific [^{32}P] DNA probes. The probe for actin mRNA was cDNA for chick actin; and for collagen, $alpha_2$ type I mouse collagen DNA.

Assay of p21 protein: Cells were grown to approximately 80% confluency in DMEM containing 10% fetal calf serum in 60 mm dishes. After washing with PBS, the cells were incubated for 16 hours in Dulbecco's medium without methionine, 2% dialized fetal calf serum, and 100 uCi/ml ^{35}S-L-methionine (Amersham) (2 ml of medium per plate). The medium was removed and the cells washed with cold PBS. One millilter of lysing buffer (0.02 M Tris pH 7.4, 5 mM $MgCl_2$, 0.1 M NaCl, 1% Triton X-100, and 0.5% deoxycholate) was added to each plate. The lysates were centrifuged for 30 minutes at 4°C at 150,000 x g, and the supernatant fluids were stored at -70°C. Aliquots of these extracts containing 5 x 10^6 dpm of TCA precipitable ^{35}S-methionine were mixed with a monoclonal antibody specific for Harvey *ras* p21 (8), and incubated for two to sixteen hours at 4°C. Fifty microliters of Staphylococcus protein A, (10% suspension)

coated with rabbit anti-rat IgG, were added and the incubation continued for another two hours at 4°C. The reactions were centrifuged at 2300 rpm for five minutes at 4°C and the pellets washed 3 or 4 times with cold buffer (1% Triton x 100, 0.5% deoxycholate, 0.1% SDS, 0.01 M sodium phosphate, pH 8, 0.1 M NaCl, and 0.001 M EDTA). After the last wash, the pellets were resuspended in 25 ul of SDS sample buffer (100 mM Tris, pH 6.8, 2% SDS, 2% mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue). The precipitates were boiled for three minutes and centrifuged at 15,000 rpm for two minutes to remove the pellet before SDS-polyacrylamide gel electrophoresis. Discontinuous 8-20% gradient slab gel electrophoresis of proteins with a 3% stacking gel was carried out (9). Gels were fixed in 7.5% acetic acid, 10% TCA, and 30% methanol for 30 minutes at room temperature. The gels were then rinsed twice with H_2O and soaked for one hour in 1M sodium salicylate. Finally, the gels were dried on a slab gel drier (Bio Rad) at 70°C for two hours. Dried gels were then autoradiographed on Kodak X-Omat film at -70°C. Quantification of p21 was carried out in a microdensitometer. The amount of p21 in revertants was compared to the amount in autoradiographs of serial dilutions of p21 from parental RS 485 transformed cells.

RESULTS: We have studied the effect of 200 units ml^{-1} of IFN on cloned RS 485, an established cell line of NIH 3T3 transformed by transfection with a human c-Ha-*ras1* oncogene activated by an Ha-MuSV LTR. The RS 485 was carried in the presence of IFN for 10-20 cell doublings, when we observed flat revertant colonies in cultures seeded at a low density (0.5 to 1% of the colonies). The frequency of reversion increased to approximately 10% by the time 35 to 40 doublings had been reached. Colonies of flat cells were isolated and clonal lines of revertant cells established from single cell cultures. No flat revertant colonies were seen in cloned RS 485 that had not been treated with IFN.

In order to test whether the transfected c-Ha-*ras* DNA was present in the revertant cell lines derived from IFN-treated RS 485 cells, we selected two of the cloned lines (4C-3 and 4C-8) and extracted high molecular weight DNA from them for restriction analysis. We employed Southern blot analysis (Fig. 1) using an Ha-*ras1* probe on EcoRI restriction fragments of 20 ug of DNA from NIH 3T3 (lane 1), cloned parental RS 485 (lane 2), revertant 4C-3 (lane 3), or revertant 4C-8 (lane 4) cells. Both 4C-3 and 4C-8 retained the transfected oncogene DNA, unchanged in quantity or distribution as compared to parental RS 485 cells, even though the biological properties of the revertant cells resembled those of nontransformed cells.

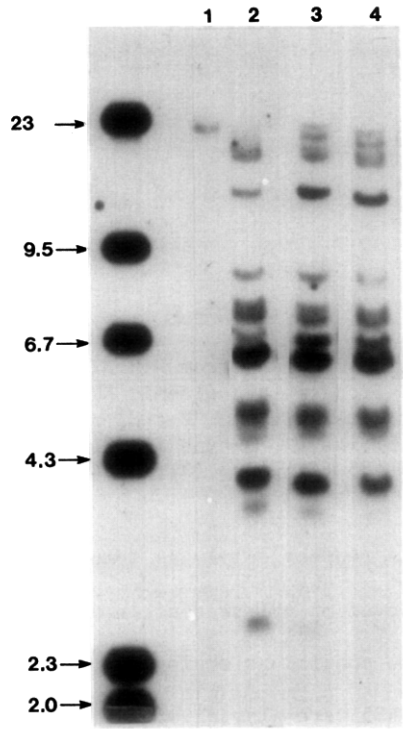

Figure 1: Southern blot analysis of DNA from NIH 3T3, RS 485, or revertant cells. 20 ug of EcoRI-digested DNA was analyzed by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-c-Ha-rasl (SacI 3kb fragment) probe. DNA from: (1) NIH 3T3; (2) RS 485; (3) 4C-3; and (4) 4C-8. Arrows are DNA markers (length in kbp).

We have also estimated the amount of c-Ha-ras specific mRNA present in the RS 485 revertants 4C-3 and 4C-8. Dot blot analysis of c-Ha-ras mRNA indicated that this RNA was consistently reduced by about 4-fold in revertant 4C-3 and 4C-8 as compared to the quantities present in the parental transformed RS 485 (Fig. 2A). There were no differences in the amounts of collagen (Fig. 2C) or actin-specific mRNA (Fig. 2B) among the different cell lines studied.

Finally, we quantified the amount of the 21 kd protein (p21) encoded by the c-Ha-rasl oncogene in the different cell lines under study. We immunoprecipitated c-Ha-ras p21 from NIH 3T3, RS 485, 4C-3 and 4C-8 with monoclonal antibody to p21. We then quantified the amounts specifically precipitated on polyacrylamide gels by scanning the autoradiographs in a microdensitometer. Our results (Fig. 3) also indicated an approximately 4-fold reduction in the amount of p21 present in the revertant cell lines 4C-3 and 4C-8 (approximately

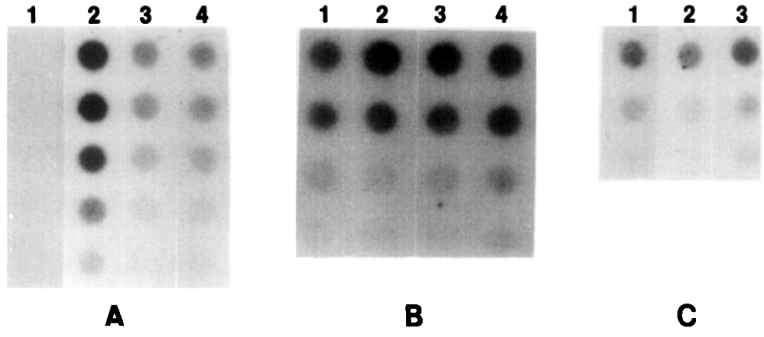

Figure 2: Dot blot analysis of mRNA from NIH 3T3, RS 485, or revertant cells. 10 ug and 1:2 serial dilutions of cytoplasmic RNA were spotted onto nitrocellulose paper and hybridized with a ^{32}P-c-Ha-ras1 (SacI 3kb fragment) probe (A); a ^{32}P-actin probe (B); or a ^{32}P-type I collagen probe (C). Cytoplasmic RNA from NIH 3T3 (1); RS 485 (2); 4C-3 (3); or 4C-8 cells (4).

20,000 and 19,000 OD units respectively), as compared to p21 present in RS 485 (86,000 OD units). The level of p21 present in untransformed NIH 3T3 cells was undetectable under the conditions employed. Immunoprecipitates (with anti-p21 antibody) of RS 485 were also diluted 1:2 and 1:4 in cell extract and treated as were the samples in Figure 3. Quantification of the OD of the p21 in the 1:2 dilution indicated the presence of approximately 35,000 OD units, while in the 1:4 dilution contained about 17,000 OD units.

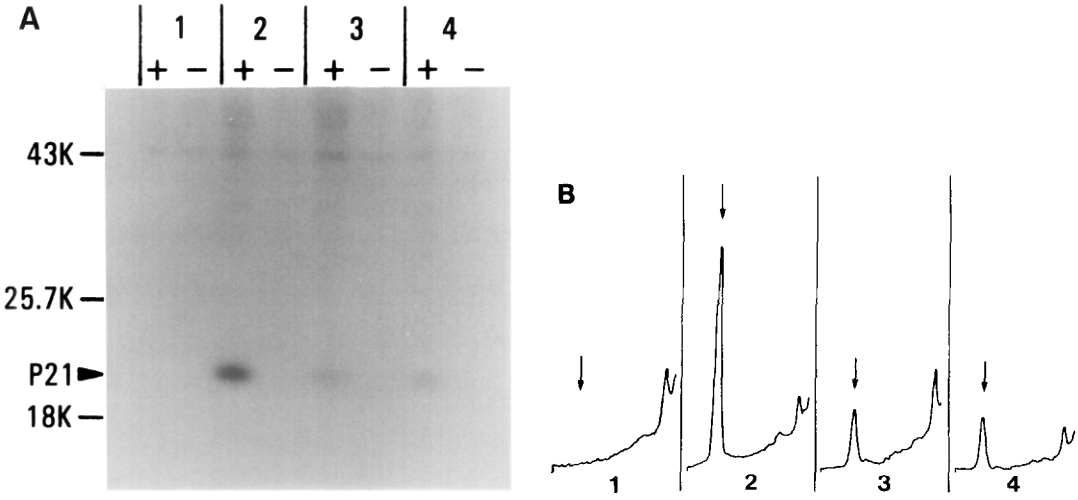

Figure 3: Quantification of ras p21 in NIH 3T3, RS 485, or revertant cells. 5 x 10^6 cpm of TCA precipitated, ^{35}S-methionine labelled cell extract was immunoprecipitated with (+) or without (-) monoclonal antibody to p21 and then subjected to SDS-polyacrylamide gel electrophoresis (A). The autoradiograph was scanned in a microdensitometer (B). The arrows indicate the position of p21. (1) NIH 3T3; (2) RS 485; (3) 4C-3; and, (4) 4C-8.

DISCUSSION: With prolonged treatment of ras-oncogene transformed RS 485 cells, IFN induced reversion to a non-transformed phenotype with respect to cellular morphology and growth (3). The isolated revertant clonal lines (cell line 4C-3 and 4C-8) contained c-Ha-ras DNA in approximately the quantities and the genomic distribution present in parental RS 485 cells; however, both c-Ha-ras mRNA and p21 were present in reduced amounts in the reverted cells. A reduction in mRNA is not a general effect of IFN treatment in this system, since the levels of actin and type I collagen mRNA were unchanged in revertants (Fig. 2).

One important question raised by this study is the quantity of p21 (or other oncogene products) required for ras-induced transformation. Since nontransformed NIH 3T3 and many other normal cells ordinarily make small amounts of p21, there must be a concentration of p21 above which transformation takes place; therefore, it might be possible to cause phenotypic reversion of the transformed state to a nontransformed state by a biological response modifier, such as an IFN, that in some manner decreases the level of c-ras expression. These results may have some bearing on the possible use of IFNs in the treatment of cancer, because current findings strongly indicate that an alteration in the expression of oncogenes could be an important factor in oncogenesis (2,10-12).

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