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Suppression of Tumorigenicity in Somatic Cell Hybrids Does not Involve Quantitative Changes in Transcription of Cellular Ha-ras, Ki-ras, myc, and fos Oncogenes

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The transcriptional activity of ten cellular oncogenes was analyzed in somatic cell hybrids that had been obtained after fusion of tumorigenic Chinese hamster cells and normal mouse fibroblasts. The hybrids showed either the tumorigenic or the nontumorigenic phenotype (suppression of tumorigenicity). Out of ten c-onc genes analyzed, four (c-Ha-ras, c-Ki-ras, c-myc, and c-fos) were found to be transcriptionally active at similar levels in tumorigenic as well as in nontumorigenic (suppressed) hybrids. Thus we conclude that suppression of tumorigenicity in Chinese hamster × mouse somatic cell hybrids does not correlate with quantitative changes in expression of these cellular oncogenes. The remaining six cellular oncogenes (c-abl, c-erb A and B, c-fes, c-myb, and c-sis) were not transcriptionally active in these hybrids.

Key words: protooncogenes, tumor suppression, somatic cell hybrids, transcription

Tumorigenicity is reduced or even abolished in many somatic cell hybrids generated in vitro after fusion of tumorigenic cells with normal cells (suppression of tumorigenicity; for review, see [1]). The molecular mechanisms of suppression of tumorigenicity in somatic cell hybrids are as yet unknown. Suppression of transformed and tumorigenic phenotypes is speculated to be caused by expression of "suppressor genes" or "antioncogenes." These genes could be structural genes, whose products directly affect proliferation of cells, or regulatory genes, which function by suppression of transforming gene products. Reexpression of transformed and tumorigenic phenotypes in derivatives of the originally nontransformed (suppressed) hybrids has been correlated with the loss of specific chromosomes or

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combinations of two or more chromosomes of the normal parent (for review, see [1]). One may hypothesize that phenotypic tumor suppression is due to quantitative changes in expression of cellular oncogenes. If this hypothesis is correct, one would expect a high expression in tumorigenic parental cells and somatic cell hybrids and a reduced or largely impaired expression in suppressed hybrids. Candidate oncogenes for such alterations are the ras, myc, and fos protooncogenes, the transcriptional activity of which has been reported to be higher in malignant than in normal tissue [2,3]. Amplified oncogenes found in many tumor cells are also abundantly expressed at the RNA level (for review, see [4]). Furthermore, the Ha-ras protooncogene is able to induce oncogenic transformation in established NIH/3T3 cells when expressed at a high level [5,6].

Previously we described the suppression of the transformed and tumorigenic phenotype in somatic cell hybrids of spontaneously transformed, malignant Chinese hamster cells and normal mouse fibroblasts [7,8]. In this article we compare the level of transcription of ten different cellular oncogenes in these nontumorigenic somatic cell hybrids, in their tumorigenic derivatives, and in the parental cells. The transcript abundance of c-Ha-ras, c-Ki-ras, c-myc, and c-fos oncogenes was unaltered in transformed and suppressed somatic cell hybrids, whereas c-abl, c-fes, c-erb A and B, c-myb, and c-sis transcriptional activities were not detected.

MATERIALS AND METHODS

Cell Culture and Isolation of Somatic Cell Hybrids

Interspecific hybrids were generated by fusion of tumorigenic Chinese hamster cell lines (Wg3-h-o, CI-4, TK17-0, and E 36-o) with early passage BALB/c mouse embryonic fibroblasts as described previously [7,8]. The phenotypes and chromosomal constitutions of these hybrids have been described in detail [8]. Mouse chromosomes were identified by sequential staining with Giemsa (G-banding) and Hoechst 33258. For each hybrid clone, 20 or more metaphase spreads were analyzed. Cells were scored positive when at least 10% of the metaphases contained the particular chromosome. The presence in the hybrids of mouse chromosomes 6, 7, 12, and 15, to which cellular ras, fos, and myc oncogenes had been assigned, was confirmed by detection in cell extracts of the mouse isozymes triosephosphate isomerase (EC 5.3.1.1), glucosephosphate isomerase (EC 5.3.1.9), acid phosphatase 1 (EC 3.1.3.2), and superoxide dismutase 1 (EC 1.15.1.1), respectively [8,9].

Northern Blot Analysis

Total cellular RNA was prepared from hybrid clones and parental cells by using the guanidine isothiocyanate method [10,11]. RNA was fractionated by electrophoresis through 1% agarose 2.2 M formaldehyde gels for 18 hr at 50 V and subsequently transferred to nitrocellulose filters [12]. Filters were hybridized with ^{32}P-labeled oncogene-specific DNA fragments. Prehybridization was performed at 42°C for 16 hr in 50% (v/v) formamide, 5× SSC (1× SSC: 0.3 M NaCl, 0.03 M sodium citrate), 5× Denhardt's reagent, 50 mM Na_2 HPO_4/NaH_2PO_4, pH 6.5, 0.1% SDS, and 250 μg/ml salmon sperm DNA. Hybridization (36 hr at 42°C) was carried out in 50% (v/v) formamide, 5× SSC, 4× Denhardt's reagent, 20 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 0.1% SDS, 100 μg/ml salmon sperm DNA, and 10% (w/v) dextran sulfate

containing the labeled probe. Filters were washed with 2× SSC, 0.1% SDS at room temperature for 30 min, followed by 0.1× SSC, 0.1% SDS at 60°C for 60 min.

Cytoplasmic Dot Blot Hybridization

Logarithmically growing cells (1–10 × 10^6) were trypsinized, harvested by centrifugation, counted, and lysed in 0.5% (v/v) Nonidet P-40 (Roth, Karlsruhe, FRG) as described [13]. Nuclei were removed by centrifugation (15,000g, 2.5 min). Nuclei-free extracts were denatured by incubation in the presence of 7.5% (v/v) formaldehyde in 15× SSC at 60°C for 15 min, and afterwards frozen at −70°C. Prior to hybridization analysis, extracts were thawed and serially diluted with 15× SSC in a 96-well microtiter plate to yield the indicated cell numbers in a final volume of 50 μl. Diluted extracts were dotted onto Biodyne A nylon filters (Pall, Glen Cove, NY) using a Manifold SRC-96 apparatus (Schleicher and Schuell, Keene, NH). Nylon filters were hybridized with a β actin probe to confirm the appropriate dilution of cytoplasmic extracts (data not shown) and with oncogene-specific DNA fragments. Washed filters were exposed to Kodak XAR-5 films in the presence of intensifier screens for 48 hr at −70°C.

Oncogene Probes

The following DNA fragments were prepared from plasmids, ^{32}P-labeled by nick translation (specific activity ⩾ 1 × 10^8 cpm/μg), and used as probes for the detection of transcripts related to cellular oncogenes: v-Ha-ras (BS9, 0.46 kb BglI/SalI fragment), v-Ki-ras (HiHi3, 0.8 kb HincII fragment), v-myc (1.5 kb PstI fragment), v-fos (1.3 kb BglII/PvuII fragment), v-sis (0.9 kb PstI/XbaI fragment), v-erb A and B (2.5 kb PvuII fragment), v-fes (0.5 kb PstI fragment), v-myb (HAX 4, 1.0 kb HaeI/XbaI fragment), and v-abl (pAB3 Sub3, 1.2 kb SmaI/BglII and 0.8 kb BglII fragments).

RESULTS

Following somatic hybridization of malignant Chinese hamster cell lines with early passage mouse embryo fibroblasts, hybrids were isolated that exhibited either expression or nonexpression (suppression) of tumorigenicity in nude mice as well as of proliferation in semisolid agar medium, respectively. The phenotype and chromosomal constitution of these hybrids has been described in detail [7,8]. Tumorigenic parental cells and somatic cell hybrids formed tumors in nude mice after subcutaneous injection of less than 50 cells. Their cloning efficiency in semisolid agar medium ranged between 6 and 40%. Compared to these highly malignant cells, suppressed hybrids required a 100- to 50,000-fold inoculum of cells to initiate tumor growth in nude mice, and the latency periods were three- to sixfold longer. Similarly, the ability to proliferate without anchorage was found to be decreased (cloning efficiency in semisolid agar medium ranged from 0.2% to 0.01%). To determine the transcriptional activity of Ha-ras, Ki-ras, myc, and fos protooncogenes in hamster × mouse hybrids, total cellular RNA was prepared from cells in logarithmic growth phase and subjected to Northern blot analysis using v-Ha-ras-, v-Ki-ras-, v-myc-, and v-fos-specific DNA fragments as radioactive probes. Transcripts of expected size were detected (Fig. 1): 1.4 kb (c-Ha-ras); 5.2, 2.0, and 1.2 kb (c-Ki-ras); 2.7 kb (c-myc); and 3.5 and 2.0 kb (c-fos). The predominance of the 2.0 kb c-Ki-ras transcript relative to the other

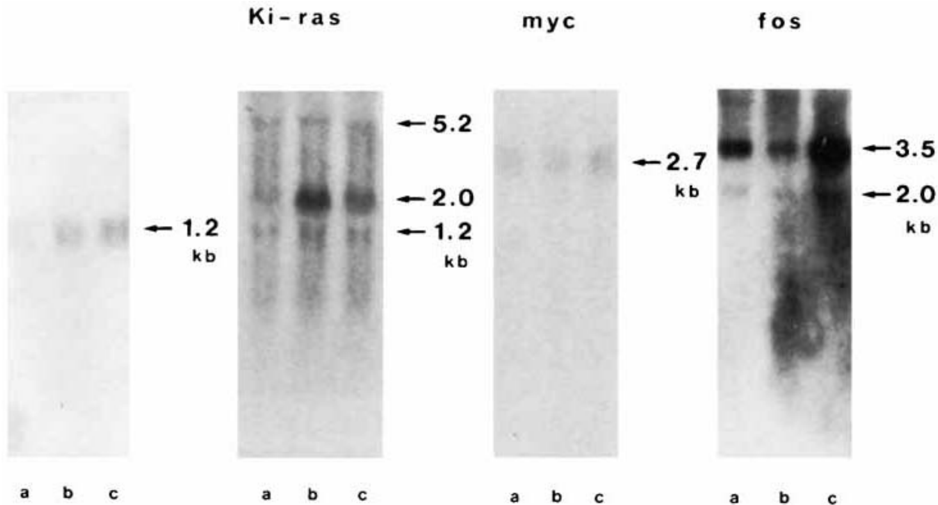

Fig. 1. Size of c-onc transcripts in Chinese hamster × mouse somatic cell hybrids. Northern blot analysis of total RNA from clones 50 BW-6 (**a**), 50 BW-6T (**b**), and 50 BW-6-1a (**c**) as described in Materials and Methods.

two transcripts was not constantly observed in all hybrids. The 3.5 kb transcript related to c-fos represents an unspliced mRNA precursor [14].

We then compared relative mRNA levels of these protooncogenes by dot blot hybridization of cytoplasmic extracts [13] from hybrids and parental cells using the same probes. All cytoplasmic cell extracts were prepared from logarithmically growing hybrid and parental cells. To compare the transcript abundance of c-onc genes in different clones, aliquots of cellular extracts corresponding to 2.5×10^5, 1.25×10^5, 6×10^4, and 3×10^4 cells from each clone were dotted onto nylon filters as described in Materials and Methods. Transcripts of c-Ha-ras, c-Ki-ras, c-myc, and c-fos were detectable in extracts corresponding to as few as 3×10^4 cells (Table I). As an example, hybridization of dotted extracts from parental and hybrid cells with a v-Ki-ras probe is shown in Figure 2. By cytoplasmic dot hybridization, transcripts of c-abl, c-erb A and B, c-fes, c-myb, and c-sis were not detectable in extracts corresponding to 2.5×10^5 or less cells.

Equal levels of mRNA related to c-Ha-ras, c-Ki-ras, c-myc, and c-fos were found in suppressed hybrid clones isolated early after fusion and in tumor outgrowths derived from them (Table I). The transcript abundance of these cellular oncogenes was also unchanged in another set of somatic cell hybrids (clones 2W3, 2W6, 2W14, and 6W4), which were as tumorigenic as the parental hamster cells. The suppressed hybrid clone 50 BW-6 was exceptional in that it had lower mRNA levels related to c-Ha-ras, c-Ki-ras, and c-myc than the other suppressed hybrids. Cells from a tumor outgrowth (50 BW-6T), however, showed again the same transcript abundance as the other hybrids. In general, mRNA levels related to c-Ha-ras, c-Ki-ras, c-myc, and c-fos frequently appeared to be at least twofold elevated in hybrids compared to the parental cells (Table I; exception: 50 BW-6). This result is probably explained by the fact that the hybrids contain a near-tetraploid or near-hexaploid hamster genome as well as a different number of mouse chromosomes [7,8].

The technique used did not allow us to distinguish directly between protooncogene transcripts of hamster or mouse origin. To find out whether the transcriptional

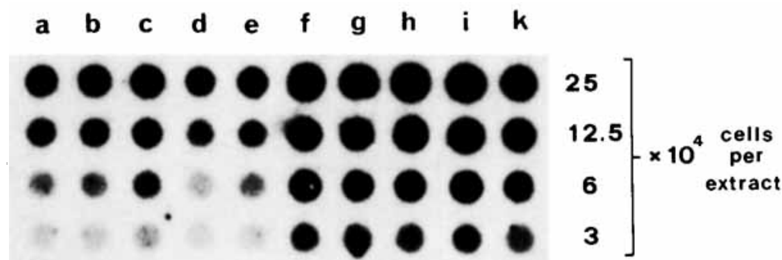

Fig. 2. Cytoplasmic dot hybridization of cellular extracts with a Ki-ras probe. Preparation of cellular extracts and conditions of dot hybridization are as described in Materials and Methods. Probe: 0.8 kb HincII fragment of plasmid HiHi3 (v-Ki-ras), ^{32}P-labeled by nick translation (specific activity $\geqslant 1 \times 10^8$ cpm/μg).**a–d**) Tumorigenic Chinese hamster cell lines Wg3-h-o, CI-4, TK 17-o, and E 36-o, respectively; **e**) normal embryonic fibroblasts; **f–h**) tumorigenic Chinese hamster × mouse hybrids 2W3, 2W6, and 2W14, respectively; **i**) nontumorigenic (suppressed) Chinese hamster × mouse hybrid 20 BW-4; **k**) 20 BW-4T, a cell population isolated from a tumor derived after subcutaneous injection of 1×10^7 cells of clone 20 BW-4 into a nude mouse (latency period, 30 days).

activity of mouse protooncogenes contributed significantly to the overall expression found in the hamster × mouse hybrids, we compared transcript levels related to c-Ha-ras, c-Ki-ras, c-fos, and c-myc in those hybrids that had either lost or retained the mouse chromosomes, to which the corresponding protooncogene loci had been assigned. The c-Ha-ras, c-Ki-ras, c-fos, and c-myc loci have been assigned to mouse chromosomes 7, 6, 12, and 15, respectively [15–18]. As shown by karyotypic analysis, these mouse chromosomes were retained in hybrid clones 20 BW-4, 50 BW-12, and 2W23, whereas the other hybrid clones had either lost one (eg, clone 2W3), more than one (eg, clone 20 BW-4T), or all of them (eg, 2W14) (Table II). In cellular extracts prepared from clones that had lost a particular mouse chromosome we detected only the hamster isozyme activities of triosephosphate isomerase (gene locus assigned to mouse chromosome 6), glucosephosphate isomerase (mouse chromosome 7), acid phosphatase 1 (mouse chromosome 12), and superoxide dismutase 1 (mouse chromosome 15). Furthermore, there was no evidence of consistent chromosomal translocations involving any of these mouse chromosomes in the hybrids [7–9]. Despite the loss of any of these mouse c-onc loci, the hybrid clones showed the same overall transcriptional activity of protooncogenes as did hybrid clones that had retained the corresponding mouse chromosomes (cf. Table I).

DISCUSSION

The comparison of oncogene transcription in tumor tissue with normal tissue is hampered not only by the heterogeneity of the tumor biopsy material but also by the presence of different cell types in the corresponding normal tissue. Nontumorigenic (suppressed) hybrids and their tumorigenic segregants are advantageous for such a comparative analysis in that they resemble clonal cell populations. In addition, their tumor-forming capacity can be exactly quantitated by injecting decreasing numbers of cells into immunosuppressed animals [cf. 8]. In human tumors transcriptional activity of c-Ha-ras, c-Ki-ras, c-myc, and c-fos oncogenes was frequently elevated compared to normal tissue [2,3]. We present evidence that the transcriptional activity of these cellular oncogenes was equal in tumorigenic and suppressed hybrids between

TABLE I. Relative mRNA Levels Related to Cellular Oncogenes in Tumorigenic and Nontumorigenic Chinese Hamster × Mouse Hybrids and Their Parental Cells

Cells	Phenotype	c-Ha-ras	c-Ki-ras	c-myc	c-fos	c-abl, c-erb A/B, c-fes, c-myb, c-sis
Parental cells						
Wg3-h-o	T	+	+	+	+	−
CI-4	T	+	+	+	+	−
TK-17-o	T	+	+	+	+	−
E 36-o	T	+	+	+	+	−
Embryonic fibroblasts	N	+	+	+	+	−
Somatic cell hybrids						
50 BW-6	N	+	+	+	++	−
50 BW-6T	T	++	++	++	++	−
50 BW-12	N	++	++	++	++	−
50 BW-12T	T	++	++	++	++	−
20 BW-4	N	++	++	++	++	−
20 BW-4T	T	++	++	++	++	−
2W23	N	++	++	++	++	−
2W23T	T	++	++	++	++	−
2W3	T	++	++	++	++	−
2W6	T	++	++	++	++	−
2W14	T	++	++	++	++	−
6W4	T	++	++	++	++	−

Preparation of cellular extracts, conditions of dot hybridization, and oncogene probes as described in Materials and Methods. Intensities of dot hybridization were compared by visual inspection of X-ray films and with the help of a laser densitometer (2202 Ultroscan, LKB, Bromma, Sweden): +, transcripts detected in cell extracts corresponding to 3×10^4 cells; ++, at least twofold intensity of hybridization; −, no hybridization found. T, tumorigenic phenotype; N, nontumorigenic or partially suppressed phenotype expressed in the indicated hybrid clones or parental cells as described in the text. Hybrids 20 BW-4T, 50 BW-6T, 50 BW-12T, and 2W23 T were isolated from tumorigenic cell populations obtained after subcutaneous injection into nude mice of clones 20 BW-4, 50 BW-12, and 2W23 (1×10^7 cells), respectively.

tumorigenic Chinese hamster cells and normal mouse cells. Furthermore, decreased rates of cell proliferation frequently found in suppressed hybrids [cf. 8] were not reflected in an appropriate decrease in transcript abundance of protooncogenes.

The overall mRNA levels related to c-Ki-ras, c-Ha-ras, c-fos, and c-myc were unaltered in those hybrids that had apparently lost the corresponding mouse structural genes as compared to those that still had retained them. Possibly, in hamster × mouse hybrids that retained mouse chromosomes 6, 7, 12, and 15, expression of the corresponding mouse c-oncogenes is suppressed. By analysis of polypeptides expressed in hamster × mouse hybrids (clones 20 BW-4 and 2W23) and separated by two-dimensonal gel electrophoresis, it has been demonstrated previously that the genome of the tumorigenic hamster parent can extinguish expression of a substantial number of mouse genes [9]. In the exceptional hybrid clone 50 BW-6, transcript levels related to protooncogenes were as low as in the parental cells. This clone has a modal number of 32 Chinese hamster and 22 mouse chromosomes [cf. 8]. A possible explanation is that there are trans-acting genes present in the mouse genome that down regulate the level of protooncogene expression in this hybrid clone. The loss of such regulatory genes together with the duplication of the Chinese hamster chromo-

TABLE II. Loss and Retention of Mouse Chromosomes With c-onc Gene Loci in Chinese Hamster × Mouse Hybrids

	Retention of mouse chromosomes with assigned c-oncogene loci			
Hybrid cells	6[a]	7[b]	12[c]	15[d]
20 BW-4	+	+	+	+
20 BW-4T	+	+	−	−
50 BW-6[e]	+	+	+	ND
50 BW-12	+	+	+	+
50 BW-12T	+	−	−	ND
2W23	+	+	+	+
2W23T	+	+	−	+
2W3	+	+	+	−
2W6	+	+	−	+
2W14	−	−	−	−
6W4	−	−	+	−

All hybrids exhibited equal mRNA levels related to c-Ha-ras, c-Ki-ras, c-fos, and c-myc (cf. Table I). Chromosome analysis and isozyme determinations were as described in Materials and Methods. +, Mouse chromosome found in at least 10% of metaphases analyzed, mouse isozyme activity detected in cell extracts; −, mouse chromosome not detected, isozyme activity not found. ND, karyotype not analyzed.

[a–d]Mouse chromosomes to which c-Ki-ras, c-Ha-ras, c-fos, and c-myc genes, respectively, have been assigned.

[e]Individual mouse chromosomes were not identified in hybrid 50 BW-6T (tumorigenic derivative of 50 BW-6).

some complement may result in an at least twofold increase in the level of protooncogene expression found in a tumorigenic segregant (50 BW-6T).

DNA from the tumorigenic hamster parental cells was used to transfect preneoplastic mouse NIH/3T3 cells. However, no activated oncogene capable of transforming these recipient cells with high efficiency was detectable (R. Schäfer, M. Dubbert, and K. Willecke, unpublished results). Other cellular oncogenes or yet unidentified transforming genes may directly contribute to expression of the transformed phenotype in the parental Chinese hamster cells and their corresponding somatic cell hybrids. The structure and function of putative suppressor genes contributed by the genome of normal cells in a hybrid genome are still unknown [cf. 1]. We favor the hypothesis that these suppressor genes may function via mechanisms not altering expression of oncogenes, eg, by inhibition of the function of oncogene products. This interpretation can be reconciled with the findings that tumorigenicity is suppressed in somatic cell hybrids even in the presence of activated cellular oncogenes [19–22] or viral oncogenes [23,24].

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