Decreased *c-myc* expression is an early event in retinoic acid-induced differentiation of F9 teratocarcinoma cells

(oncogene/malignancy/leukemia)

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ABSTRACT Retinoic acid-induced differentiation of mouse F9 teratocarcinoma cells is known to be accompanied by changes in gene expression. We examined the expression of the *c-myc* gene during retinoic acid-induced differentiation of F9 cells. Retinoic acid caused a 50% reduction in the level of *c-myc* mRNA after 3 hr of treatment and a 90% reduction after 12 hr. We have also examined several features of *c-myc* genomic structure in F9 cells, PYS2 (differentiated parietal yolk sac) cells, and liver—namely, methylation, amplification, and gross organization. Comparison of *Hpa* II and *Msp* I digests of DNAs from F9, PYS2, and liver showed that the *c-myc* gene in F9 cells is hypomethylated relative to that in PYS2 cells and in liver. The *Hpa* II sites that undergo methylation during differentiation were found to be in the second intron. Gross structural changes of the *c-myc* gene have not occurred in F9 or PYS2, and the *c-myc* gene is not amplified.

Retinoic acid has been shown to be a potent inducer of the *in vitro* differentiation of several malignant stem cell lines to benign, terminally differentiated cells. This process is characterized by loss of the rapid proliferation typical of malignant cells and by changes in gene expression. F9 teratocarcinoma cells can be induced to differentiate with physiological levels of retinoic acid in a dose-dependent fashion (1) into parietal or visceral endoderm (1–3). Changes in gene expression have been demonstrated during the F9 differentiation process; however, most of the changes associated with identified genes occur sometime after the actual induction of differentiation and appear to be structural in nature (1, 3, 4).

To address the mechanism of differentiation, we have examined the effect of retinoic acid on the expression of the oncogene *c-myc*. The *c-myc* gene product is known to be induced after growth stimulation of both T and B lymphocytes and of fibroblasts (5). Malignancies of the hematopoietic system in mouse and human, plasmacytomas and lymphomas, contain structurally altered *c-myc* genes that are expressed at high levels (6, 7). The *c-myc* gene, which is amplified and highly expressed in HL-60 cells, is down-regulated during retinoic acid-induced differentiation of these cells (8–10).

When F9 teratocarcinoma cells are treated with retinoic acid, dibutyryl cAMP and theophylline for 3 days, the level of *c-myc* mRNA is reduced (11). In light of the evidence for the antiproliferative and differentiative actions of retinoic acid, we have investigated whether retinoic acid alone causes a decrease in *c-myc* expression in F9 cells, whether the decrease in expression in F9 cells occurs during the early stages of the differentiation process, and whether the structure of the *c-myc* gene shows any of the previously mentioned structural abnormalities.

MATERIALS AND METHODS

Cell Lines and Animals. F9 and PYS2 (1) cells were obtained from B. Kahan (Zoology Research, University of Wisconsin-Madison), and BALB/c 3T3 clone A31 was from the American Type Culture Collection. F9 and PYS2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 15% heat-inactivated fetal bovine serum, and BALB/c 3T3 cells in DMEM with 10% calf serum. Differentiation of F9 cells was induced by adding 1 μ M all-*trans*-retinoic acid in ethanol as a 1:1000 dilution into culture medium. Control cultures received 0.001 volume of ethanol. Differentiation was determined by morphological examination (1) and by immunoprecipitation of laminin from the culture medium of retinoic acid-treated cells (2), using medium conditioned by F9 cells and PYS2 cells as negative and positive controls, respectively. Liver was obtained from a 2-month-old male mouse of the A/J strain.

Plasmids and Probe Preparations. Strains containing the following plasmids were obtained: mouse *c-myc* pM104BH (6), from J. Gorski (Dept. of Biochemistry, University of Wisconsin-Madison); mouse α -globin pseudogene p30.5 (12), from O. Smithies (Dept. of Genetics, University of Wisconsin-Madison); mouse *c-fos* (13), from R. Risser (Dept. of Oncology, University of Wisconsin-Madison); and *v-erbB* (14) and *v-Ha-ras* (15), from H. Pitot (Dept. of Oncology, University of Wisconsin-Madison). Plasmids were prepared according to standard protocols. Gene-specific DNA was released by appropriate restriction enzyme digestion; the fragments were purified by electrophoresis in agarose gels, and DNA was recovered by electroelution. The pM104BH clone contains *c-myc*-specific DNA from the first intron, second exon, second intron, and third exon of the gene and is 3.2 kilobases (kb) long (Fig. 3E). Probes specific for different portions of the gene were generated by double digestions, as shown in Fig. 3E.

RNA Isolation. RNAs were isolated from F9, retinoic acid-treated F9, PYS2, and 3T3 cells by the method of Strohman (16). RNA was quantified by absorbance at 260 nm. Messenger RNA was purified by chromatography on oligo(dT)-cellulose (17). Messenger RNA fractions were quantified using absorbance at 260 nm and binding to poly([³H]uridylic acid) (18).

Isolation of High Molecular Weight Eukaryotic DNA. DNA was isolated from F9 and PYS2 cells by the method of Blin and Stafford (19). A nuclear fraction of mouse liver was prepared by homogenizing the tissue in 50 mM Tris Cl, pH 7.5/5 mM MgCl₂/0.2% Triton X-100 in a Dounce homogenizer and recovering the nuclear fraction by centrifugation at 12,000 $\times$ g. DNA was then isolated as described for cell lines. DNA was quantified by absorbance at 260 nm.

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Abbreviation: kb, kilobase(s).

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Nick-Translation. Agarose gel-purified fragments used as probes were labeled to a specific activity of $1-3 \times 10^8$ dpm/ μ g, using 200 pmol of [α - 32 P]dATP and of [α - 32 P]dCTP (Amersham) per μ g of DNA, according to Maniatis *et al.* (17). The labeled probe was separated from unincorporated radio-labeled deoxynucleotide by Sephadex G-50 spin-column chromatography (17).

Southern Blot Hybridization. After digestion with the relevant restriction endonuclease, genomic DNA samples (10 μ g) were electrophoresed in 0.8% or 1.2% agarose gels and transferred to nitrocellulose according to the method of Southern (20). To determine completeness of restriction enzyme digestion, samples of genomic DNA plus bacteriophage λ DNA were digested at the same DNA/enzyme ratio and concentrations were as samples for Southern analysis. These parallel digests were analyzed in agarose gels for the presence of the expected λ fragments on a background smear of eukaryotic DNA. Fragments of *c-myc* plasmids were included on gels at 30 pg, 10 pg, and 1 pg as hybridization controls and autoradiography markers. λ HindIII and pBR322 HinfI digests were used as gel markers and excised prior to transfer. Blots were baked *in vacuo* at 80°C for 2 hr, then prehybridized for 6 hr in 50% formamide at 42°C according to Wahl *et al.* (21). Hybridizations were carried out as described (21) and included 10% dextran sulfate. Blots were washed in $2 \times$ SSC/0.1% NaDodSO₄ at room temperature and then in $0.1 \times$ SSC/0.1% NaDodSO₄ at 65°C. ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate.) After drying, the blots were exposed to Kodak XAR5 film at -70°C, using Kodak intensifying screens. Blots were erased for reuse in 5 mM Tris Cl, pH 7.4/2 mM EDTA/2 $\times$ Denhardt's solution/0.1% NaDodSO₄ at 75-80°C for 3 hr. ($1 \times$ Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.)

DNA Dot Blot Hybridization. Samples (10 μ l) of serially diluted DNAs were boiled 2 min in 0.2 M NaOH and then cooled on ice for 5 min. Cold 8 M ammonium acetate was added to 1 M final concentration. After samples were spotted onto a nitrocellulose filter equilibrated in 1 M ammonium acetate, the filter was air-dried, soaked in 2 $\times$ Denhardt's solution for 1 hr, air-dried, and then baked, hybridized, washed, and exposed as for Southern blots.

Blot Hybridization of Electrophoretically Fractionated RNA. Samples (5 μ g) of poly(A)⁺ RNA were boiled for 5 min in sample buffer (50% formamide/2.2 M formaldehyde/0.5 $\times$ Mops gel buffer), cooled on ice for 5 min, electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose, and baked for 3-4 hr at 80°C *in vacuo* (17). Filters were prehybridized and hybridized for 6 and 18 hr, respectively, according to Wahl *et al.* (21), in 50% formamide and 10% dextran sulfate at 42°C. Filters were washed first in $2 \times$ SSC/0.1% NaDodSO₄ at room temperature and then in $0.1 \times$ SSC/0.1% NaDodSO₄ at 65°C. Filters were dried and exposed as for Southern blots.

RNA Dot Blot Hybridization. Poly(A)⁺ RNA samples were boiled in 0.44 M formaldehyde/3 $\times$ SSC and then cooled on ice for 5 min. Serial dilutions (1:2) were made into cold 20 $\times$ SSC and 10- μ l samples were spotted onto nitrocellulose equilibrated in 20 $\times$ SSC (22). Filters were then prepared as for other blots. Hybridizations and washes were as described above, except that the final wash for blots to viral oncogene probes was in $1 \times$ SSC/0.1% NaDodSO₄ at 50°C.

RESULTS

Decrease in *c-myc* Expression in Differentiating F9 Cells. To study the effect of retinoic acid on *c-myc* expression, F9, PYS2 (differentiated parietal yolk sac), and 3T3 (nontumorigenic) cells were harvested at mid-log phase and cytoplasmic poly(A)⁺ RNA was prepared from each. Cultures of F9 cells

at mid-log phase were also treated with 1 μ M all-*trans*-retinoic acid for 3, 6, 9, 12, 24, or 48 hr before harvest. The poly(A)⁺ RNA samples were fractionated by electrophoresis and analyzed for *c-myc* mRNA by blot hybridization (Fig. 1A). A decrease in the levels of *c-myc* mRNA was detectable by 3 hr after treatment with retinoic acid. There is one *c-myc* mRNA species present in F9, in retinoic acid-treated F9, and in PYS2 cells, with an approximate size of 2.1 kb, as judged by the migration of denatured *c-myc* DNA fragments run as hybridization controls and autoradiography markers. More lengthy exposures of blots showed a second mRNA species, at approximately 2.5 kb, in 3T3 cell mRNA. The autoradiograph in Fig. 1A was scanned by densitometry and the results (Fig. 1B) indicate that there is a 46% decrease in *c-myc* mRNA in F9 cells by 3 hr after retinoic acid treatment. This decrease continues for the first 12 hr after treatment, to a level 10% of that in F9 cells. The amount of *c-myc* mRNA in F9 cells is approximately 25 times more than in 3T3 cells, and the amount of *c-myc* mRNA in PYS2 cells is about 25% that in F9 cells. The decrease in *c-myc* mRNA was also demonstrated by RNA-DNA dot blot hybridization under the same conditions (data not shown).

Changes in Expression of Other Cellular Oncogenes. Since there is evidence that expression of some oncogenes changes during development (22), changes in expression of *c-fos*, *c-erbB*, and *c-Ha-ras* during F9 differentiation were investigated. The same mRNA samples used for the *c-myc* analysis shown in Fig. 1 were used for RNA dot blot analysis and hybridized to probes for the *c-fos*, *v-erb*, and *v-Ha-ras* genes. Fig. 2 shows the results of quantitation of these dot blots. The level of *c-erbB* message increased almost 4-fold at 9 hr after induction of differentiation, and the level of *c-fos* message increased 2- to 3-fold starting at 12 hr after induction. The level of *c-Ha-ras* message did not change during differentiation.

Methylation State of Genomic DNA in F9 Versus Differentiated Cells. It has been shown that the potential for gene expression correlates with the degree of methylation of a gene and its surrounding DNA sequence (23). Since the levels of *c-myc* mRNA decrease during differentiation of F9 cells, the methylation state of the *c-myc* gene in F9 cells and in differentiated cells was investigated. *Hpa* II and *Msp* I digests of F9, PYS2, and liver DNA were electrophoresed in 1.2% agarose gels, transferred, and hybridized. When the nitrocellulose filter was probed with the 3.2-kb probe that covers the *c-myc* gene from the middle of the first intron through the 3' untranslated region, fragments of sizes 6.3, 0.7, 0.5, and 0.4 kb were recognized (Fig. 3A). Since bands were not identified at 6.3 kb and 0.7 kb in *Hpa* II-digested liver or PYS2 DNA but were identified in *Hpa* II-digested F9 DNA, sites contributing to these fragments must be methylated in liver and PYS2 DNA but not in F9 DNA.

The portion of the gene containing the methylated sites was identified by hybridization of the same filter to subfragments of the 3.2-kb probe (Fig. 3B). The 1.5-kb *Sst* I-*Hind*III probe, which covers the second intron and third exon of the gene, is homologous to the sites that have different methylation states in these DNAs, fragments of 6.3 and 0.7 kb. The 0.9-kb *Xba* I-*Sst* I second-exon probe hybridized to the 0.5-kb fragment (Fig. 3C), which therefore must arise at least in part from *Hpa* II restriction sites in the second exon. Hybridization to the *Sst* I-*Xba* I first-intron probe (Fig. 3D) identifies the same fragment as the second-exon probe (Fig. 3C), indicating that the fragment spans the region delineated by these two probes, the first intron and the second exon. Finally, the *Sst* I-*Xba* I probe does not recognize the 0.4-kb fragment, indicating that this fragment must be homologous, in part at least, to the extreme 5' end of the pM104BH probe, the *Bam*HI-*Sst* I probe (data not shown). Thus, the *c-myc* gene in the F9 DNA is hypomethylated with respect to both that in the PYS2 DNA

FIG. 1. (A) Analysis of poly(A)⁺ RNA in F9 and retinoic acid-treated F9 cells by hybridization with nick-translated 3.2-kb *Bam*HI-*Hind*III *c-myc* probe. Electrophoresis, transfer, and hybridization are described in *Materials and Methods*. (B) Quantitation of decrease in *c-myc* mRNA with retinoic acid treatment of F9 cells. The autoradiograph in A was scanned on a Zeineh soft laser scanning densitometer (Biomed Instruments, Fullerton, CA). For each sample, relative peak areas were determined and that value was used to calculate the amount of *c-myc* mRNA present in samples from retinoic acid-treated F9 cells (and from untreated PYS2 and 3T3 cells; data not plotted) relative to the amount present in samples from untreated F9 cells.

and that in the liver DNA. Fig. 3E summarizes the positions of these *Hpa* II sites in the gene.

Gross Structural Organization of *c-myc* in F9 and PYS2 DNA. The organization of the *c-myc* gene in F9 and PYS2 DNA was examined for large abnormalities such as translocations, deletions, or insertions. In mouse plasmacytoma *c-myc* genes, chromosomal translocations of *c-myc* coding sequences to immunoglobulin regulatory regions results in

aberrant regulation of expression of *c-myc* and the affected immunoglobulin gene (24). To investigate the gross genomic structure of the *c-myc* gene in F9 and PYS2, DNA from F9, PYS2, and mouse liver was analyzed by Southern blotting. *Bam*HI digests generated one fragment for each DNA, with the same approximate size of 5.6 kb; *Hind*III digests generated one fragment, with a size of 4.6 kb; and the *Eco*RI digests generated one fragment, with a size of 22 kb (Fig. 4). These results indicate that there are no gross deletions or insertions within the portions of the gene identified by these digests.

Amplification of the *c-myc* Gene in F9 and PYS2 DNA. The *c-myc* gene in HL-60 cells is amplified and highly expressed but is down-regulated during retinoic acid-induced differentiation (8-10). It was therefore of interest to determine whether the *c-myc* gene in F9 cells is amplified. The genomic Southern blot in Fig. 4 was erased and reprobed with the p30.5 gene, a single-copy mouse α -globin pseudogene (12). The relative intensity of hybridization of the p30.5 probe to liver vs. F9 and PYS2 DNA (data not shown) is the same as that of the *c-myc* probe (Fig. 4). This indicates that the *c-myc* gene is not amplified in F9 and PYS2 DNA relative to liver DNA. DNA dot blot analysis confirmed that there is no difference in *c-myc* copy number between F9, PYS2, and liver DNAs (Fig. 5).

FIG. 2. Quantitation of changes in mRNA levels of oncogenes during F9 cell differentiation. Serial dilutions of poly(A)⁺ RNA samples (1, 0.5, 0.25, and 0.125 μ g) from F9 cells and F9 cells treated with retinoic acid for 3, 6, 9, 12, 24, or 48 hr were spotted onto nitrocellulose and hybridized with *c-Ha-ras* (●), *c-erbB* (■), or *c-fos* probe (▲). For each probe, hybridization controls (100, 50, 25, and 12.5 pg of probe) were spotted. The resulting autoradiographs were scanned on a Zeineh soft laser scanning densitometer; relative peak areas were determined and that value was used to calculate the percent of oncogene mRNA of untreated F9 cells present in samples from retinoic acid-treated F9 cells.

DISCUSSION

The results show that the down-regulation of *c-myc* expression in F9 cells occurs within 3 hr of retinoic acid treatment. To our knowledge, this is the earliest reported change in gene expression during F9 cell differentiation. This result is consistent with the hypothesis that regulation of the *c-myc* gene is a key event in this differentiation. Second, the level of mRNA in the F9 stem cell is 25 times greater than in nontumorigenic control 3T3 cells. This figure is consistent with previous estimates of a 20-fold elevation of the *c-myc* mRNA level in malignant, hematopoietic stem cells over control (6, 7, 24). Third, the data show the presence of one mRNA species (2.1 kb) in all cases except control 3T3 cells,

FIG. 3. Southern blot analysis of *c-myc* methylation patterns in F9, PYS2, and mouse liver genomic DNA. (A–D) DNA samples were digested with *Hpa* II or *Msp* I and analyzed by Southern blot hybridization using the 3.2-kb *Bam*HI–*Hind*III *c-myc* probe (A), the 1.5-kb *Sst* I–*Hind*III *c-myc* probe (B), the 0.9-kb *Xba* I–*Sst* I *c-myc* probe (C), and the 0.5-kb *Sst* I–*Xba* I *c-myc* probe (D). (E) Diagram of the locations of the *Hpa* II sites in the *c-myc* gene giving rise to the fragments identified in (A–D). The identified fragments, as well as the probes used, are indicated in relation to restriction sites and coding sequences of the gene. B, *Bam*HI; H, *Hind*III; R, *Eco*RI; S, *Sst* I; X, *Xba* I; Hp, *Hpa* II; Hp*, methylated *Hpa* II sites. Black bars represent regions; open bars, 3' untranslated region; bp, base pairs.

where two are found [2.1 kb (the predominant species) and 2.5 kb].

The expression of several other oncogenes during F9 cell differentiation was found to be altered during the first 12 hr of F9 differentiation (Fig. 2). The amount of *c-erbB* mRNA increased 4-fold between 6 and 9 hr after induction. An increase in *c-erbB* expression was expected, since the level of epidermal growth factor receptor, coded for in part by the *c-erbB* gene, is known to increase with retinoic acid treatment of F9 cells (25). The *c-fos* mRNA level increases

FIG. 4. Southern blot analysis of the *c-myc* gene in F9, PYS2, and mouse liver genomic DNA. DNA samples digested with *Bam*HI, *Hind*III, or *Eco*RI were analyzed by Southern blot hybridization using the 3.2-kb *Bam*HI–*Hind*III *c-myc* probe.

between 9 and 12 hr after induction and ultimately rises 3-fold. Increases in *c-fos* expression have also been documented during differentiation (26). When mRNA samples from F9 and retinoic acid-treated F9 cells were probed for *c-Ha-ras* expression, no difference was found during the early period of differentiation. This is in agreement with previously reported data on *c-Ha-ras* expression during development (22). Although the responses of these genes to retinoic acid in this system was expected, the early timing of the changes in *c-fos* and *c-erbB* expression suggests that they are also integral parts of this differentiation program.

We examined several aspects of the structure of the *c-myc* gene that may be involved in regulation of expression. We examined methylation patterns in the *c-myc* gene from F9

FIG. 5. DNA dot blot analysis of *c-myc* gene copy number in F9, PYS2, and mouse liver DNAs. Dilution numbers 1–5 represent, respectively, 1, 0.5, 0.25, 0.125, and 0.0625 μ g of DNA from F9, PYS2, and liver samples and 100, 50, 25, 12.5, and 6.25 pg of the 3.2-kb *Bam*HI–*Hind*III *c-myc* fragment. Equivalence of the DNAs had been determined by ethidium bromide staining of 0.3% agarose gels. Dot blot preparation and hybridization with the 3.2-kb *c-myc* probe were as described in *Materials and Methods*.

cells and their prototype differentiated derivative cell line PYS2. As a differentiated-cell control, we used DNA from mouse liver. When *Hpa* II and *Msp* I digests of these DNAs were probed for *c-myc* sequences, we found that all identified fragments in F9 DNA arose from unmethylated sites, whereas two of these fragments in both PYS2 and liver DNAs contained methylated cytidine residues (Fig. 3). Given the decrease in *c-myc* mRNA in retinoic acid-treated F9 cells and the differences in methylation between the F9 and PYS2 *c-myc* genes, it is reasonable to postulate that this gene becomes methylated during F9 cell differentiation. Reprob- ing, with subfragments of the 3.2-kb *c-myc* probe, showed that the sites that are methylated in the PYS2 and liver DNA must be located within the second intron of the gene.

The *c-myc* gene in F9 cells is not amplified relative to that in liver (Fig. 5). This is unlike the case in HL-60 cells, another retinoic acid-responsive cell line, where the *c-myc* gene is amplified 20-fold. Therefore, the high level of *c-myc* mRNA present in F9 cells must be due to either differences in *cis* or *trans* regulation of the gene or a difference in metabolism of the mRNA itself. Thus, the physiological response of decreased *c-myc* expression, which is shared by retinoic acid-treated F9 and HL-60 cells, is not related to increased *c-myc* copy number.

Structural analysis of the *c-myc* gene in F9 and PYS2 cells (Fig. 4) indicates that the translocations common in murine plasmacytomas and in human lymphomas probably have not occurred. Such gene rearrangements lead to alterations in the size of restriction fragments carrying the gene, as visualized by hybridization to this 3.2-kb probe: a large decrease in size in the *Eco*RI fragment, from 22 kb to 16 kb, and frequent changes in sizes of fragments generated by digestion with *Bam*HI and *Hind*III. In the case of F9 and PYS2, there is no difference in the sizes of fragments generated by digestion with *Bam*HI, *Hind*III, and *Eco*RI relative to liver, and the size estimates of these fragments are in agreement with known sizes for fragments of the normal murine *c-myc* gene. Therefore, any changes that may exist in this gene are too small to be detected by this approach.

We have demonstrated that the down-regulation of the *c-myc* gene is one of the earliest observed events in retinoic acid-induced F9 cell differentiation. Since the protein product of this gene is thought to play a regulatory role in cell growth, we have identified one of the first known effects of retinoic acid in F9 cell differentiation that is likely to be involved in the differentiation itself. The molecular mechanism of this response is unknown, but further study may contribute to understanding the mechanism of action of retinoic acid in cell differentiation.

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