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Antisense *Myc* sequences induce differentiation of F9 cells

(oncogene/retinoic acid)

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ABSTRACT **Down-regulation of *Myc* expression is the earliest documented change in gene expression in retinoic acid-induced differentiation of murine F9 teratocarcinoma cells. F9 cells transfected with plasmids expressing antisense *Myc* sequences under control of the simian virus 40 (SV40) early promoter exhibit a decrease in Myc protein. The result of this decrease is the spontaneous differentiation into cells that resemble retinoic acid-treated F9 cells as judged by plasminogen activator assays. In contrast, when F9 cells are transfected with a plasmid expressing *Myc* under control of the SV40 early promoter, resulting cell clones are resistant to differentiation by retinoic acid as shown by the lack of induction of plasminogen activator. These results suggest that down-regulation of *Myc* is sufficient and necessary for F9 cell differentiation.**

The murine teratocarcinoma cell line, F9, differentiates from malignant stem cells to benign extraembryonic parietal endoderm when treated with physiological doses of all-*trans*-retinoic acid (1). This differentiation process is marked by dramatic changes in the cell's phenotype, reflective of the many differences in gene expression between F9 and differentiated F9 cells. Among these changes are increases in expression of plasminogen activator and laminin (1, 2) and a decrease in expression of stage-specific embryonic antigen 1, SSEA-1 (3). To study the mechanism of action of retinoic acid in induction of differentiation, expression of various cellular protooncogenes has been monitored in F9 and differentiating F9 cells. The earliest identified change was the decrease of Myc mRNA within 3 hr of retinoic acid induction (4).

Down-regulation of *Myc* expression also accompanies retinoic acid-induced human HL-60 cell differentiation (5) and dimethyl sulfoxide-induced murine MEL cell differentiation (6). Further, it has been shown that constitutive expression of *Myc* in MEL cells by the use of surrogate promoters inhibits the ability of dimethyl sulfoxide to induce differentiation (7, 8). Such data suggest that down-regulation of *Myc* is necessary for differentiation.

We have asked if down-regulation of *Myc* expression is sufficient as well as necessary for differentiation of F9 cells. To address this question, we have introduced plasmids expressing antisense Myc RNAs into F9 cells. Utilization of plasmids to express antisense RNAs is thought to be capable of suppressing gene function by the formation of RNA·RNA hybrids in the nucleus, which then inhibit transport of RNA to the cytoplasm, inhibit protein translation, and/or increase RNA turnover (9, 10). Several recent experiments, including the suppression of cytokeratin endo B in retinoic acid-treated F9 cells (11) and the inhibition of growth stimulation in quiescent murine 3T3 cells by expression of antisense Fos mRNA (12), indicate that use of antisense-RNA-generating plasmids can successfully block expression of the desired product. Our transfection results indicate that introduction of such plasmids leads to spontaneous differentiation. Additionally, transfection of F9 cells with a plasmid expressing sense *Myc* under control of a surrogate promoter results in cell clones that are resistant to differentiation by retinoic acid. Thus, our data suggest that down-regulation of *Myc* in F9 cells is sufficient and necessary for induction of differentiation.

MATERIALS AND METHODS

Cell Growth, Differentiation, and Transfection. F9 cells were grown and treated with all-*trans*-retinoic acid as described (4). Transfections were performed by the calcium phosphate precipitation method (13) as follows: cells were plated at 1×10^6 per 100-mm dish or 3×10^6 per 150-mm dish 1 day prior to transfection; transfections contained 10 μg of specified DNA and 3 μg of pCDneo (14) and were incubated for 20 hr. Transfected cells were then divided into two portions and grown in the presence of 400 μg of G418 (GIBCO) per ml for periods indicated in the text.

Plasminogen Activator Assays. The plasminogen activator overlay assays (15) were performed with 133 μg of plasminogen (Sigma) per ml in 1:1:1 (vol/vol) 8% (wt/vol) nonfat dry milk/Dulbecco's modified Eagle's medium/2.5% (wt/vol) purified agar in phosphate-buffered saline. Quantitation of plasminogen activator activity was performed by using the ^{125}I-labeled fibrin–Linbro plate radioassay (15). Incubations were for 3–5 hr at 37°C.

Plasmid Constructions. The DNA plasmids, pMyc2.3, pAntimyc0.4, and pAntimyc2.3, were constructed by using standard cloning techniques as elaborated in the text. The modified Okayama–Berg cloning vector p1151 and the plasmid CW1-28 were obtained from Paul Lambert (National Cancer Institute), pCDneo was obtained from Hiroto Okayama (National Cancer Institute), and the *Myc* clone pR*S10 was obtained from Kenneth Marcu (State University of New York-Stonybrook).

Isolation of DNA and Southern Analysis. Genomic DNA was isolated from cell clones by the method of Gross-Bellard *et al.* (16). Southern blot analysis was performed as described (4).

Isolation of RNA, Primer Synthesis, and Primer Extension Analysis. Total RNA was isolated from cell clones by the procedure of Chirgwin *et al.* (17). Quantitative primer extension analysis was performed as described by Phelps and Howley (18), and the products were resolved by electrophoresis on 6% (wt/vol) polyacrylamide/8 M urea gels. The primer for pMyc2.3 was 5′-TTGGTGAAGTTCACGTT-GAGGGG-3′ and is complementary to the murine Myc RNA beginning 41 nucleotides downstream from the start of exon 2 of the gene (19). The primer used to identify endogenous transcripts was 5′-GAGCGCGGCTCTGCACACACGGC-3′ and is complementary to the endogenous RNA starting 77

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Abbreviation: SV40, simian virus 40.

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nucleotides downstream from the start site of promoter P_2 and 240 nucleotides downstream from the start site of P_1 (19).

Immunoblotting. Cells were lysed in a minimal volume of radioimmunoprecipitation assay buffer [50 mM Tris·HCl, pH 7.4/150 mM NaCl/1% (wt/vol) Triton X-100/1% (wt/vol) sodium deoxycholate/0.1% $NaDodSO_4$/0.3 mg of phenylmethylsulfonyl fluoride per ml] on ice for 30 min. The lysates were electrophoresed on 7.5% (wt/vol) $NaDodSO_4$/polyacrylamide gels, electrotransferred, and blotted with antibody by using the Vectastain ABC method (Vector Laboratories, Burlingame, CA). The affinity-purified rabbit anti-human MYC antibody was provided by Takis Papas (National Institutes of Health, Frederick, MD). Analysis of Myc levels in transfected cells was performed at various times after transfection to determine maximal down-regulation.

RESULTS

Construction of Antisense *Myc* Plasmids. Since down-regulation of Myc mRNA occurs very soon after induction of F9 differentiation with retinoic acid, it was of interest to determine if this is sufficient to induce differentiation. The approach used was to determine what effect transfection of plasmids expressing antisense Myc RNA had on the phenotype of F9 cells. Two different regions of the murine *Myc* gene were cloned downstream of the simian virus 40 (SV40) early enhancer/promoter in modified Okayama–Berg cDNA expression vectors. The SV40 early enhancer/promoter region, resident in these vectors, is known to express in both undifferentiated and differentiated F9 cells (20). The vector CW5-1/Bam was created by deletion of the cDNA sequences from CW1-28 (21). A 0.4-kilobase (kb) fragment containing exon 1 of the murine *Myc* gene from the plasmid pR*S10 (22) was cloned as a *Bam*HI fragment into the unique *Bam*HI site in CW5-1/Bam in the antisense orientation to result in the plasmid called pAntimyc0.4. A 2.3-kb fragment containing exons 2 and 3 and intron 2 [the *Xba* I–*Xho* I 2.3-kb fragment from the plasmid pM104BH (4)] was cloned into the unique *Xho* I site in p1151 (21) in the antisense orientation, resulting in the plasmid pAntimyc2.3 (Fig. 1*A*).

Decrease in Myc Protein in F9 Cells Transfected with pAntimyc Plasmids. To determine whether transfection of the pAntimyc plasmids causes a decrease in Myc protein, pAntimyc0.4 and pAntimyc2.3 were each cotransfected with pCDneo into F9 cells and maintained under positive selection for G418 resistance. Cells were harvested 10 days after the initial addition of G418, and protein lysates were immunoblotted for Myc protein. The Myc proteins, identified as two species of approximately 65 and 62 kDa, were seen in the lysate from cells transfected with the SV40 cloning vector, p1151, and pCDneo (Fig. 1*B*, lane 3) but were substantially reduced in the lysate from cells transfected with pAntimyc0.4 and pCDneo (lane 4). A similar decrease in Myc levels was seen in lysates from cells transfected with pAntimyc2.3 and pCDneo (data not shown). The level of Myc proteins in lysates from untransfected F9 cells was similar to that in p1151 control transfections (Fig. 1*B*, lane 1), and a marked decrease in Myc proteins was seen in lysates from retinoic acid-treated F9 cells (Fig. 1*B*, lane 2). Attempts were made to detect antisense RNA in such transfected cell populations; however, convincing data were not obtained. Based upon previous reports, antisense RNA is likely unstable and therefore difficult to detect (9, 12). As there was no obvious decrease in the level of endogenous sense Myc RNA in cells transfected with either pAntimyc plasmid (data not shown), we believe that the decrease in Myc is due to either inhibition of RNA transport or protein translation but not to increase in RNA turnover. These results establish that pAntimyc plasmids cause a decrease in the levels of Myc when transfected into F9 cells.

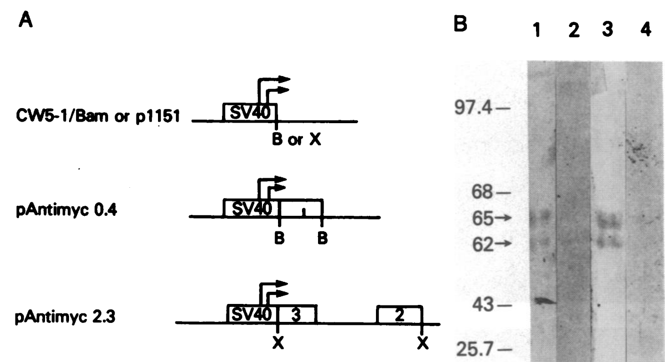

FIG. 1. Analysis of Myc protein in cells transfected with pAntimyc plasmids. (*A*) Description of pAntimyc0.4 and pAntimyc2.3. Derivation of pAntimyc clones is diagramed, showing the insertion of murine *Myc* sequences from pR*S10 or pM104BH in the antisense orientation into the modified Okayama–Berg cloning vectors CW5-1/Bam or p1151, respectively. See Fig. 3*A* for a diagram of the murine *Myc* gene. B, *Bam*HI; X, *Xho* I. (*B*) Immunoblot analysis of Myc proteins. Ten micrograms of protein was electrophoresed. Lysates in lanes are from F9 cells (lane 1), retinoic acid-treated F9 cells (lane 2), cells transfected with p1151 and pCDneo (lane 3), and cells transfected with pAntimyc0.4 and pCDneo (lane 4). Protein molecular mass standards are indicated on the left; arrows indicate the sizes of the Myc protein bands, 65 and 62 kDa.

Differentiated Phenotype of F9 Cells Transfected with pAntimyc Plasmids. We utilized a biochemical assay, the plasminogen activator overlay assay, to look for differentiation of F9 cells after transfection with pAntimyc plasmids. This assay follows for identification *in situ* of cells producing plasminogen activator, a protein produced and secreted in large amounts by differentiated F9 cells but not by undifferentiated F9 cells. F9 cells, cotransfected with pCDneo and one of pAntimyc0.4, pAntimyc2.3, or p1151, were overlaid with casein–agar containing plasminogen on day 5 or 7 after initiation of G418 selection. Fig. 2 shows representative overlays of control F9 cells treated with 0.5 μM all-*trans*-retinoic acid for 72 hr (Fig. 2*A*) and cells cotransfected with p1151 and pCDneo (Fig. 2*B*), pAntimyc0.4 and pCDneo (Fig. 2*C*), or pAntimyc2.3 and pCDneo (Fig. 2*D*). Large numbers of plaques were clearly visible on plates treated with retinoic acid or transfected with pAntimyc plasmids. Few plaques were seen on plates transfected with the vector p1151. Overlays of plates containing cells transfected with a variety of other control plasmids did not show plaques (data not shown). Populations of antisense plasmid-transfected cells showed increased laminin production and decreased SSEA-1 production by immunostaining (data not shown), consistent with the conclusion that the pAntimyc plasmids induced differentiation. Cotransfection of F9 cells with pCDneo and either pAntimyc plasmid was found to result in an average 14-fold increase in the number of plaques over that obtained by cotransfection with p1151 and pCDneo (Table 1). The low incidence of plaque formation after cotransfection with p1151 and pCDneo and after G418 selection indicates that the low density of surviving cells that would result from this protocol is not sufficient to efficiently induce differentiation. When these control transfections were treated with retinoic acid prior to overlay, high numbers of plaques similar to that obtained by pAntimyc transfection were seen (Table 1). This indicates that the number of surviving cells in control versus pAntimyc transfections is similar. Furthermore, this comparison shows that differentiation by pAntimyc transfection is a high-frequency event. These results establish that, under the conditions of this assay, down-regulation of *Myc* is sufficient for inducing differentiation of F9 cells at a high efficiency.

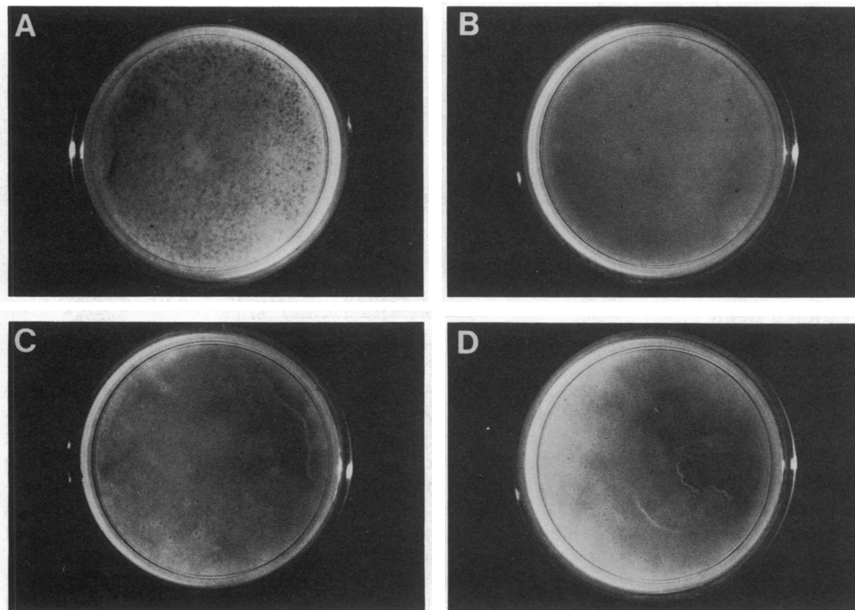

FIG. 2. Plasminogen activator overlay analysis of pAntimyc-transfected F9 cells. (*A*) Overlay on F9 cells treated with 0.5 μM all-*trans*-retinoic acid for 72 hr. (*B*) Overlay on cells cotransfected with p1151 and pCDneo. (*C*) Overlay on cells cotransfected with pAntimyc-0.4 and pCDneo. (*D*) Overlay on cells cotransfected with pAntimyc2.3 and pCDneo.

Production of Stable Cell Clones Expressing pAntimyc Plasmids. To determine if only a small percentage of F9 cells was differentiating in response to pAntimyc action, numerous attempts were made to establish clonal cell lines of G418-selected cells from cotransfection experiments with antisense plasmids and pCDneo. Of the 25 colony clones examined, none contained the transfected antisense DNA in orientations preserving the SV40 promoter–antisense gene integrity and, in fact, most clonal lines entirely lacked the antisense *Myc*-transfected gene (data not shown). By contrast, in cotransfection experiments with either the vector or the sense clone, pMyc2.3 (see following section), the majority of clones raised contained intact plasmid DNA. Furthermore, markedly fewer G418-resistant colonies were obtained in stable pAntimyc cotransfections than in control transfections after 2–3 wk of G418 selection, and a majority of these colonies were small, differentiated, and nonexpandable. Recent experiments examining the effect of antisense *Myc* oligodeoxyribonucleotides on human HL-60 cell growth and differentiation indicate that a majority of these cells differentiated in response to antisense *Myc* influence (23). The inability to obtain cell clones expressing the intact pAntimyc plasmids would be expected were differentiation the result of antisense Myc RNA action, as terminally differentiated cells soon cease to proliferate. Thus, these results would appear to discount the possibility that only a subpopulation of the transfected F9 cells is sensitive to differentiation in response to antisense Myc RNA expression because, were that the case, stable clones containing the intact antisense DNA should have been found.

Table 1. Induction of plasminogen activator activity in pAntimyc-transfected F9 cells

DNA	Number of plaques per 10^6 cells transfected*					
	Day 5 exp.		Day 7 exp.			
	1	2	1	2	3	4
p1151 + pCDneo†	4	8	20	10	10	5
pAntimyc0.4 + pCDneo	92	48	127	61	172	130
pAntimyc2.3 + pCDneo	95	36	88	71	150	149

*F9 cells (1 × 10^6) were cotransfected with the above DNAs as described in the text. The plasminogen activator overlay assays were performed at the indicated times, and the number of plaques resulting in each transfection was determined. The number of plaques indicated represents the average of 2–3 transfections for each experiment.

†When treated with 1 μM retinoic acid for 48 hr prior to overlay on day 7, the number of plaques increased to 200.

Creation of Cell Clones Expressing pMyc2.3. Since the above data suggest that down-regulation of *Myc* is sufficient for differentiation of F9 cells, we also wished to determine if down-regulation is necessary for differentiation to occur. Therefore, we asked whether constitutive expression of *Myc* in F9 cell clones inhibits the ability of retinoic acid to induce differentiation. To provide an appropriate *Myc*-containing plasmid for this study, the 2.3-kb *Xba* I–*Xho* I *Myc* fragment previously described was placed in the sense orientation in p1151 to give pMyc2.3 (Fig. 3*A*). All 5′ and 3′ *Myc* regulatory sequences and the endogenous *Myc* promoters are removed in pMyc2.3, thus placing expression under heterologous control of the SV40 promoter and polyadenylylation signals. After cotransfection of pMyc2.3 and pCDneo into F9 cells and 2 wk of selection in G418-containing medium, colonies were isolated and lines were developed. Fig. 3 shows the molecular data obtained on a sampling of such clones. Southern blot analysis of DNAs prepared from these clones using the 2.3-kb *Myc* fragment (Fig. 3 *A* and *B*) as probe identified the *Hin*dIII-digested endogenous gene as a 4.6-kb fragment (lanes 1–11) in both control clones (lanes 1–3) and in the candidate sense clones (lanes 4–11). The unit length of transfected pMyc2.3 plasmid was identified by a 5.7-kb fragment after linearization with *Hin*dIII (lane M) and was found in seven of eight pMyc2.3 clones. Primer extension analysis with an oligodeoxyribonucleotide primer for pMyc2.3 on RNA isolated from control and pMyc2.3 clones is shown in Fig. 3*C*. The predicted SV40 promoter-derived primer extension products (Fig. 3*A*) were found in all clones containing the pMyc2.3 plasmid. Fig. 3*D* shows the presence of endogenous Myc transcripts arising from the endogenous *Myc* P_2 promoter (19) in control p1151 and pMyc2.3 clones (Fig. 3*A*). The levels of endogenous transcripts were not affected by the presence of expressing pMyc2.3. Products initiated at P_1, 240 nucleotides upstream from the oligonucleotide start, were also unaffected by the presence of pMyc2.3 (data not shown). The expression of the pMyc2.3 plasmid is constitutive as the level of transcripts detected in RNA from retinoic acid-treated cells from these clones was not diminished; however, the level of endogenous Myc

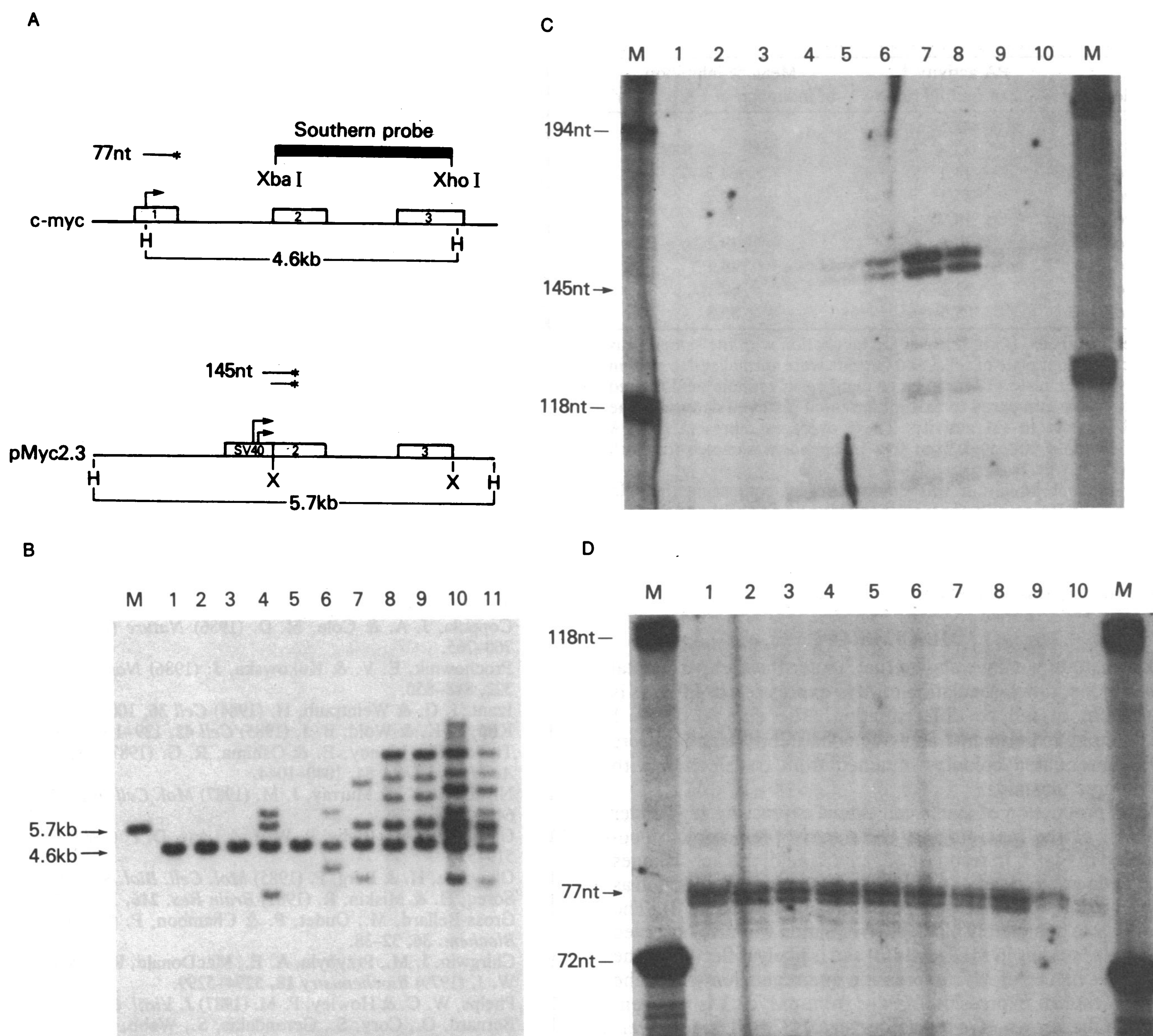

FIG. 3. Molecular analysis of pMyc2.3 cell clones. (*A*) Description of molecular tools showing the derivation of pMyc2.3 (the 2.3-kb *Myc* fragment that was both the fragment inserted into p1151 and the fragment for the hybridization probe), the positions of the oligodeoxyribonucleotide primers for pMyc2.3, and the endogenous gene used for primer extension analysis. H, *Hin*dIII; X, *Xho* I. (*B*) Southern blot analysis of sense *Myc* clones. Ten micrograms of DNAs isolated from cell clones was digested with *Hin*dIII and blotted with ^{32}P-nick-translated pMyc2.3 5.7-kb fragment shown in *A*. Lanes: M, marker (10 pg of pMyc2.3 linearized with *Hin*dIII); 1, clone containing pCDneo only; 2 and 3, clones containing p1151 and pCDneo; 4–11, clones containing pMyc2.3 and pCDneo. (*C*) Primer extension analysis of transcripts from the transfected pMyc2.3 plasmid. Quantitative primer extension analysis was performed on 20 μg of total RNA. Lanes: M, ^{32}P-labeled ϕX174 *Hae* III fragments with the indicated sizes; 1–3, clones containing p1151 and pCDneo; 4–10, clones containing pMyc2.3 and pCDneo. (*D*) Primer extension analysis of endogenous Myc transcripts from murine *Myc* promoter. Quantitative analysis was performed as in *C*. Lane identification is the same as in *C*. nt, Nucleotides.

transcripts was reduced, as previously seen (7) (data not shown).

Response of pMyc2.3 Cell Clones to Retinoic Acid. The response of the pMyc2.3 cell clones to retinoic acid was measured by the induction of plasminogen activator activity (Table 2). The pMyc2.3 cell clones analyzed in Fig. 3 were seeded at 5×10^4 cells per 30-mm dish; 24 hr later cells were fed with medium containing 0.5 μM all-*trans*-retinoic acid or an equivalent volume of ethanol. After 48 hr conditioned media were harvested and assayed for plasminogen activator activity by plasminogen-dependent solubilization of ^{125}I-labeled fibrin in the Linbro plate assay. Plasminogen activator activity increased 40-fold when normalized to protein concentration in control clones treated with retinoic acid. This increase was comparable to that originally reported (1). The average increase in plasminogen activator activity in pMyc2.3 clones examined was 15.8, or 60% less than controls (Table 2). Thus, constitutive *Myc* expression partially inhibited the action of retinoic acid in inducing differentiation of the F9 cells, reflected by a 60% decrease in plasminogen activator induction. This effect on plasminogen activator induction is specific to the pMyc2.3 plasmid. The inhibitory effect of constitutive *Myc* expression on retinoic acid-induced differentiation was further documented by determining that the production and secretion of laminin were markedly inhibited as measured by immunoprecipitation of [^{35}S]methionine-labeled laminin from conditioned medium (data not shown).

Table 2. Inhibition of retinoic acid-induced plasminogen activator (PA) activity in pMyc2.3 clones

Clone	PA activity,* mean fold increase	Mean % inhibition of induction of PA activity†
p1151-1	45.5	—
p1151-2‡	33.4	—
pMyc2.3-1	14.1	64.3
pMyc2.3-3	16.1	59.2
pMyc2.3-4	18.7	52.7
pMyc2.3-5	24.0	39.2
pMyc2.3-6	20.1	49.1
pMyc2.3-7	12.2	69.1
pMyc2.3-8	5.6	85.8

*The ^{125}I-labeled fibrin released by incubation with the sample was calculated per plate of cells, and the data were normalized to protein content per plate. The values of cpm/μg in retinoic acid-treated plates were compared to that in untreated plates to determine the fold increase in PA activity. These averages represent the PA activity data collected from five independent samples for each condition.

†The percent inhibition was determined by comparing the fold increase in PA activity for the sense pMyc2.3 clones to the average fold increase in PA activity for the control p1151 clones.

‡The two control clones are representative of six different control clones and F9 cells. The average fold increase in PA activity for all control data was 40.3 ± 7.4.

DISCUSSION

In this study we have shown that, under these experimental conditions, down-regulation of *Myc* expression in F9 cells is sufficient to induce differentiation. The data in Table 1 summarize experiments showing consistently high numbers of differentiated colonies obtained from transfection with pAntimyc plasmids.

The production of stable cell clones expressing *Myc* under control of the heterologous SV40 early promoter is documented in Fig. 3. In response to retinoic acid, this set of clones showed various degrees of resistance to retinoic acid action as measured by plasminogen activator activity (Table 2). The inability to completely block differentiation was also observed in similar studies in MEL cells (8) and is likely reflective of the complex nature of *Myc* expression and regulation within the cell. Although expression of *Myc* from pMyc2.3 is independent of the known *Myc* regulatory signals (24), it is, however, not certain that other signals do not exist elsewhere in the gene or that the level of Myc protein produced from pMyc2.3 was sufficient to completely block differentiation. Thus, this study also demonstrates that down-regulation of *Myc* is necessary for differentiation of F9 cells.

Based upon these observations, *Myc* regulation appears to play a direct role in F9 cell differentiation. The capacity for antisense *Myc* sequences to induce differentiation of F9 cells (this work) and the observation that treatment of F9 cells with retinoic acid leads to an early decrease in Myc RNA levels (4, 25) may provide important insights into the mechanism of F9 cell differentiation. Commitment to differentiation in response to retinoic acid has been shown to require cell-cycle events occurring in early S phase (26, 27). Consistent with this observation, arrest of F9 cell growth in G_1 phase, while resulting in decreased Myc levels, is not sufficient to induce differentiation (25, 27, 28). Recent reports indicate that Myc plays an important, if not direct, role in DNA synthesis (29, 30), presumably during S phase. Thus, in consideration of these data, differentiation induced by retinoic acid could occur in the following manner. A key inductive event resulting from retinoic acid treatment is the down-regulation of *Myc* expression. When the cells move into S phase, the decreased Myc level affects DNA replication in a manner which results in commitment to differentiation.

Finally, the fact that down-regulation of *Myc* is both sufficient and necessary for F9 cell differentiation raises the possibility that the gene is controlled either directly by retinoic acid-mediated action or at an early point in the differentiation program. With the recent evidence that a nuclear receptor exists for retinoic acid (31, 32), it is now reasonable to postulate direct control of gene expression by retinoic acid in differentiation, to identify genes transcriptionally regulated by retinoic acid, and to determine the mechanism of regulation of *Myc* by retinoic acid.

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