Growth Factor-Deprived BALB/c 3T3 Murine Fibroblasts Can Enter the S Phase after Induction of *c-myc* Gene Expression

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Induction of quiescent BALB/c 3T3 murine fibroblasts by platelet-derived growth factor (PDGF) or fibroblast growth factors (FGFs) is accompanied by induction of *c-myc* gene expression. To study the role of *c-myc* in cell growth, we transfected BALB/c 3T3 cells with a plasmid construct containing a glucocorticoid-inducible *c-myc* gene. When these transfected cells were growth arrested in PDGF-FGF-free defined medium, glucocorticoid treatment induced S-phase DNA synthesis. This induction of DNA synthesis was inefficient, and cell proliferation was not evident, suggesting that growth factors act through stimulation of *c-myc* expression together with other intracellular events.

The *c-myc* gene encodes a nuclear protein which is preferentially expressed during proliferation of many types of normal and neoplastic cells (1, 4, 6, 14, 19, 22). Serum, platelet-derived growth factor (PDGF), and fibroblast growth factors (FGFs) rapidly induce *c-myc* expression in quiescent fibroblasts before the onset of DNA synthesis (14). Several lines of data strongly suggest that *c-myc* induction is functionally significant. *c-myc* protein microinjection into quiescent fibroblasts induces DNA synthesis (13), and transfection of fibroblasts with *c-myc* genes linked to strong promoters improves cell growth and plating efficiency in medium supplemented with PDGF-poor plasma or low serum concentration (2).

We have determined that growth of 3T3 mouse fibroblast cells in hormonally defined culture medium absolutely requires the presence of PDGF or FGF (25). We wished to determine the role of *c-myc* expression in such cell growth. For this purpose, we transfected BALB/c 3T3 cells with a *c-myc* gene fused to a glucocorticoid-inducible promoter and monitored the cycling of these cells after culture in defined medium containing dexamethasone, but lacking PDGF or FGF. We found that dexamethasone induction of *myc* expression stimulates S-phase DNA synthesis in some cells but that significant cell proliferation does not occur. Hence, *c-myc* expression is partially, but likely not solely, responsible for growth factor induction of cell proliferation.

MATERIALS AND METHODS

Plasmid constructions. pSVMmycH is a plasmid containing murine *c-myc* cDNA fused to the mouse mammary tumor virus long terminal repeat sequence (MMTV LTR). As diagrammed in Fig. 1, pSVMmycH bears the 1.9-kilobase-pair *Hind*III DNA fragment of pMmmyc54 (20) bearing the entire translational open reading frame of *c-myc*; the 1.5-kilobase-pair *Hind*III DNA fragment of pSVMdhfr (15) containing the MMTV LTR; and the 5-kilobase-pair *Hind*III DNA fragment of pKOneo (5) containing pBR322, the simian virus 40 early promoter region, the simian virus 40 t intron,

and an RNA polyadenylation signal sequence. The 750-base-pair *Sac*I fragment of pSVMmycH was cloned into pSP64 (16) in the antisense orientation with respect to the SP6 promoter, generating pSP6-antimyc. pSV40mycH is similar to pSVMmycH, but lacks the 1.5-kilobase-pair *Hind*III fragment bearing the MMTV LTR. pEH-myc contains genomic human *c-myc* (kindly provided by F. Alt), and the *Eco*RI-*Cl*aI fragment containing exon 3 (3) was used in filter blot hybridizations. pLTRneo contains the Tn5 neomycin-G418 resistance gene fused to the Moloney murine leukemia virus LTR (25). pHu4A contains the human histone H4 gene (10).

Cell culture. BALB/c 3T3 cells were maintained in Dulbecco medium supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.). BALB/c 3T3 cells were transfected with calcium phosphate-precipitated plasmid and carrier DNAs by a standard procedure (24). For defined medium cell culture, the basal defined medium (termed DMI) contained Dulbecco medium and Ham's F12 nutrient mixture, inorganic supplements, histidine, bovine serum albumin-linoleic acid complex (Sigma Chemical Co., St. Louis, Mo.), transferrin, and insulin (Collaborative Research, Inc., Waltham, Mass.) as previously described (25). Cells were plated in serum-containing medium onto poly-L-lysine-fibronectin-coated culture dishes (Nunc, Roskilde, Denmark) at approximately 20% confluency. After cell attachment, cultures were refed with DMI and refed with DMI 12 h later, and experiments were initiated 24 h after that (termed $T = 0$ h). At $T = 0$, cultures were refed with DMI unsupplemented or containing 1 μ M dexamethasone (Sigma) or 50 ng of FGF (Collaborative Research) per ml.

TdR incorporation assays. DMI-arrested cell cultures (10,000 cells plated per 1.8-cm² culture microwell) were fed with DMI with or without dexamethasone or FGF. After 15 h, 2.5 μ Ci of [³H]thymidine ([³H]TdR) (New England Nuclear Corp., Boston, Mass.) per ml was added and incubation continued for 6 h. Cultures were washed with buffered saline, fixed on ice with 15% trichloroacetic acid, and washed with water. To measure total TdR incorporation, we treated fixed cultures with 0.5 M NaOH to dissolve DNA, which was added to Aquasol (New England Nuclear Corp.) and counted by liquid scintillation counting. For in situ

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FIG. 1. Map of plasmid pSVMmycH. The map of the *EcoRI*-linearized plasmid is shown. pSVMmycH contains murine *c-myc* cDNA (horizontally dashed box) including the complete protein-coding sequence (triple hashmarked box). The MMTV LTR (box with circles) provides the glucocorticoid-inducible promoter, which initiates transcription at the indicated rightward arrow. The box with vertical dashes contains the simian virus 40 t intron and a polyadenylation sequence, and the open box contains the simian virus 40 early promoter-enhancer. The indicated restriction enzyme cleavage sites are: E, *EcoRI*; B, *BamHI*; H, *HindIII*; S, *SacI*. The 750-base-pair (bp) *SacI* fragment was subcloned into plasmid pSP64 for experiments involving heteroduplex analyses (see text).

autoradiography, fixed cultures were overlaid with Kodak NTB2 emulsion, dried at 37°C, exposed at -80°C, and processed with Dektol (Kodak) developer. Cell containing radiolabeled DNA had darkened grains over their nuclei.

Assay for cell DNA content. DMI-arrested and induced cell cultures were trypsinized, washed in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline, and fixed by the slow addition of ethanol (85% [vol/vol] final concentration). Fixed cells were stained with 20 µg of chromomycin A3 (Sigma) per ml in 32 mM MgCl₂, and the relative DNA content was monitored by 457.9-nm light-induced fluorescence in a Becton Dickinson FACS-4 cell sorter. A total of 20,000 cells were analyzed for each sample.

Heteroduplex analysis of *c-myc* RNAs. Expression of endogenous *c-myc* and pSVMmycH genes was assayed by heteroduplex analysis (16). pSP6-antimyc was linearized with *EcoRI* and used as the template for synthesizing [³²P]UTP-labeled RNA transcripts with SP6 polymerase. Cytoplasmic RNAs (4 µg) (18) were hybridized with 100,000 cpm of antimyc RNA and then digested with RNases A and T₁, and protected RNAs were analyzed by electrophoresis through 5% acrylamide gels containing 8 M urea.

RNA filter blot hybridization. Cytoplasmic RNAs (10 µg) were subjected to electrophoresis through formaldehyde gels, transferred to nitrocellulose filters, and hybridized with ³²P-labeled DNA as originally described (21).

Immunoprecipitation of *myc* protein. Cells were growth arrested in DMI, appropriately induced with dexamethasone or FGF for 8 h, and refed with DME without methionine plus 300 µCi of [³⁵S]methionine per ml and 500 µg of bovine serum albumin-linoleate complex per ml, and cell extracts were prepared 1 h later as described elsewhere (9). Labeled cell extracts (10⁷ cpm) were reacted with rabbit antiserum raised against human *c-myc* protein (kindly provided by R. Eisenman), and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described elsewhere (9). Normal rabbit serum served as the negative control.

RESULTS

BALB/c 3T3 cells will grow in basal defined medium (DMI) only when supplemented with FGF or PDGF (25). We examined *c-myc* expression in these cells after DMI growth arrest and after stimulation with FGF or serum. *c-myc* expression was barely detectable in subconfluent DMI-arrested cells (Fig. 2, lane B) and increased substantially upon treatment with FGF or 10% calf serum (lanes A and C). The reduction in *c-myc* expression mediated by FGF-PDGF deprivation is similar to that caused by confluence and serum exhaustion (14).

To study the role of *c-myc* expression in cell growth, we constructed an inducible *c-myc* gene construct termed

pSVMmycH. As shown in Fig. 1, pSVMmycH is a mouse *c-myc* cDNA clone (20) linked to the glucocorticoid-responsive promoter in the LTR of MMTV. pSVMmycH was cotransfected into BALB/c 3T3 cells together with pLTRneo (25), a plasmid conferring mammalian cell resistance to the neomycin analog G418. Resultant G418-resistant colonies were pooled, growth arrested in DMI, and assayed for stimulation of DNA synthesis after treatment with the synthetic glucocorticoid dexamethasone. Treatment of cultures with dexamethasone for 15 h increased the amount of [³H]TdR incorporation into DNA as compared with that of parallel uninduced cultures (Table 1). Quiescent cultures derived from BALB/c 3T3 cells transfected with only pLTRneo were not stimulated by dexamethasone treatment. Hence, dexamethasone induction of DNA synthesis required prior transfection with pSVMmycH.

Individual G418-resistant clones were grown for further analysis. Several clones were identified that had one or two copies of pSVMmycH per haploid genome, as revealed by DNA filter blot hybridization (data not shown). Cultures of pSVMmycH-positive clones along with two clones lacking pSVMmycH were growth arrested in DMI and tested for stimulation of DNA synthesis by dexamethasone. pSVMmycH-positive clones showed increased DNA synthesis in response to dexamethasone, while pSVMmycH-negative clones were nonresponsive (Table 2). It is conceivable that pSVMmycH-positive clones bear elevated *myc* expression

FIG. 2. *c-myc* RNA expression in growth-arrested and growth-stimulated BALB/c 3T3 cells. BALB/c 3T3 cells were growth arrested in DMI and then refed with DMI plus 50 ng of FGF per ml, 10% calf serum, or no supplements. After 8 h, cytoplasmic RNAs were prepared, and 10 µg was subjected to formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose. The filter was hybridized with ³²P-labeled DNA containing exon 3 of human *c-myc*. RNA was from cells refed with DMI plus FGF (lane A), DMI without supplements (lane B), or DMI plus serum (lane C). The loading of nearly equivalent amounts of RNA per lane was monitored by UV-light shadowing of the gel before filter transfer. The arrowhead indicates the prominent 2.4-kilobase *c-myc* transcript.

TABLE 1. DNA synthesis in BALB/c 3T3 cell cultures transfected with pSVMmycH^a

Expt	Plasmid(s) transfected	Dexamethasone induction	Cpm of (3H)TdR incorporated ^b	Stimulation
1	pLTRneo	—	2,950 (100)	
		+	3,750 (100)	1.3
	pLTRneo + pSVMmycH	—	1,725 (100)	
		+	6,150 (150)	3.6
	pLTRneo + pSVMmycH	—	1,975 (130)	
2		+	6,900 (1,500)	3.5
	pLTRneo	—	6,000 (500)	
		+	7,250 (100)	1.2
	pLTRneo + pSVMmycH	—	3,610 (30)	
		+	12,150 (1,300)	3.4

^a BALB/c 3T3a cells (10⁶) were transfected with pLTRneo with or without pSVMmycH and selected in G418 for 12 days. G418-resistant colonies on each dish were trypsinized and pooled, and 10⁴ cells were plated onto each of four fibronectin-coated microwells (1.8 cm²). Cells were growth arrested by being maintained in defined medium (DMI) for 36 h and then refed with DMI with or without 2 μ M dexamethasone. After 14 h, [³H]TdR (2.5 μ Ci per well) was added for 6 h. Radioactivity in DNA was determined by trichloroacetic acid fixation in situ, elution of DNA with 0.5 M NaOH, and liquid scintillation counting.

^b Incorporation shown is the average from duplicated wells (standard deviation in parentheses).

^c Stimulation is the ratio of [³H]TdR incorporation with dexamethasone/rate of [³H]TdR without dexamethasone.

TABLE 2. DNA synthesis in BALB/c 3T3 clonal cell lines containing pSVMmycH or pSV40mycH

Expt	Clone	Inducer	cpm of [³ H]TdR incorporated	Stimulation	% Labeled nuclei
1	neo1	None	12,190		
		Dex	10,590		
		FGF	98,400	8.0	
	SVMmycH1	None	5,480		
		Dex	25,400	4.6	
		FGF	48,900	8.9	
	SVMmycH2	None	7,500		
		Dex	42,900	5.7	
		FGF	85,300	11.4	
	SV40mycH1	None	42,100		
		Dex	44,200		
		FGF	132,600	3.1	
2	neo2	None	10,800		
		Dex	9,660		
		FGF	354,000	32.7	
	SVMmycH2	None	10,900		
		Dex	68,800	6.3	
		FGF	87,500	8.0	
	SVMmycH3	None	11,960		
		Dex	35,200	2.9	
		FGF	78,500	6.6	
	3T3	None	2,800		<2
		Dex	2,970		<2
		FGF	215,000	76.8	80
3	SVMmycH4	None	29,900		10
		Dex	107,000	3.6	45
		FGF	187,000	6.3	85
	SVMmycH2	None	17,100		
		Dex	85,400	5.0	
		Dex + APC	370	0.02	

^a Cell clones were plated on microwells and growth arrested with DMI as described in Table 1, footnote *a*. Cells were refed with DMI with dexamethasone (Dex), dexamethasone plus aphidicolin (APC), FGF, or no supplements and labeled with [³H]TdR 14 h later. Radioactivity incorporated into DNA was assayed as described in Table 1, footnote *a*. In experiment 3, a duplicate well was trichloroacetic acid fixed and overlaid with photographic emulsion for in situ autoradiography. The percentage of labeled nuclei reflects microscopic examination of about 1,000 cells in random fields. Clones SVMmycH1 to -4 contain pSVMmycH, clone SV40mycH1 contains pSV40mycH, while neo1 and neo2 contain only pLTRneo. 3T3 is untransfected BALB/c 3T3 cells.

with or without glucocorticoid and that dexamethasone stimulates DNA synthesis by mediating other physiological events. To refute this possibility, we derived the 3T3 clone SV40mycH1, containing the plasmid pSV40mycH which constitutively expresses the *myc* gene (see below). DNA synthesis in DMI-arrested SV40mycH1 cells was not stimulated by the addition of dexamethasone (experiment 1 of Table 2).

In one DNA synthesis experiment, a duplicate of each culture was processed with photographic emulsion for autoradiography. After 5 days of exposure and development, cells which had synthesized DNA were scored by their nucleus-localized silver grains. Dexamethasone stimulation of DNA synthesis in pSVMmycH-positive cells reflected a roughly proportional increase in the percentage of cells undergoing DNA synthesis (Table 2, experiment 3).

Several findings in these TdR incorporation experiments should be noted. First, basal DNA synthesis in the absence of dexamethasone can be higher in pSVMmycH-positive cells than in normal BALB/c 3T3 cells (experiment 3 in Table 2). This may reflect basal uninduced expression of the transfected pSVMmycH gene sometimes observed (see below). Second, dexamethasone treatment of pSVMmycH-positive cells does not induce DNA synthesis as effectively as does FGF (Table 2). Last, dexamethasone stimulation of pSVMmycH-positive cells has never resulted in a significant increase in cell number.

We next measured the levels of *myc* gene expression in normal and pSVMmycH-positive 3T3 cells cultured in defined medium. Cultures of normal 3T3 cells and of the SVMmycH1 and SVMmycH2 clones (pSVMmycH positive) were growth arrested in DMI and then treated for 8 h with dexamethasone or FGF or not treated. Cytoplasmic RNAs were assayed for transcripts of pSVMmycH and endogenous *c-myc* genes by hybridization with ³²P-labeled *myc* antisense RNA followed by RNase digestion and gel electrophoresis. The *myc* antisense RNA was synthesized with SP6 polymerase from a pSP64 plasmid containing the 750-base-pair *Sac*I fragment of pSVMmycH (Fig. 1). Hybridization of this probe to transcripts initiating from the two endogenous *c-myc* promoters (14) would give protected fragments of 355 and 380 nucleotides, while hybridization of the probe to transcripts initiating from the MMTV LTR in pSVMmycH would generate a fragment greater than 600 bases.

c-myc RNA in DMI-arrested BALB/c 3T3 cells was below

FIG. 3. *myc* RNA expression in cell lines cultured in defined medium. (A) Heteroduplex analysis of *c-myc* RNA expression in pSVMmycH-transfected cells. The SVMmycH1 and SVMmycH2 clones of transfected BALB/c 3T3 cells along with untransfected 3T3 cells were growth arrested in DMI and refed with DMI with or without dexamethasone or FGF. Cytoplasmic RNAs were extracted 8 h later, and 4 μ g of each RNA was hybridized with 100,000 cpm of 32 P-labeled RNA synthesized from *Eco*RI-linearized pSP6-antimyc with SP6 polymerase. Reaction mixtures were digested with RNases A and T₁, and protected fragments were analyzed by electrophoresis through a polyacrylamide-urea gel. Normal 3T3 cells were refed without supplements (lane a), with dexamethasone (lane b), with FGF (lane c), or with both (lane d); SVMmycH2 cells were refed without supplements (lane e), with dexamethasone (lane f), with FGF (lane g), or with both (lane h); lane i shows a hybridization reaction without cytoplasmic RNA; SVMmycH1 cells were refed without supplements (lane m) or with dexamethasone (lane n); lane o shows normal 3T3 cells plus FGF. Lane j contains undigested probe, and lanes k and l contain 32 P-labeled ϕ X174 *Hinf*I-digested DNA fragments, whose nucleotide lengths are indicated. The lower arrowheads denote the smaller of two RNase-protected fragments corresponding to endogenous *c-myc* RNA, and the upper arrowheads denote pSVMmycH RNA. (B) Analysis of *c-myc* expression in pSVMmycH- and pSV40mycH-transfected cells by RNA filter blot hybridization. The SVMmycH2 and SV40mycH1 clones along with untransfected BALB/c 3T3 cells were arrested and refed with DMI with or without dexamethasone. RNA was prepared from cells 8 h later and assayed for *c-myc* transcripts as described in the legend to Fig. 2. RNA was prepared from normal 3T3 cells (lanes a and d), SVMmycH2 cells (lanes b and e), and SV40mycH1 cells (lanes c and f) after culture with dexamethasone (lanes d to f) or without (lanes a to c). Transcripts from pSVMmycH, pSV40mycH, and endogenous *c-myc* are all approximately 2.4 kilobases in length.

detection at this length of autoradiographic exposure (Fig. 3A, lane a). Expression was substantially increased by treatment with FGF (lane c) but not dexamethasone (lane b). Glucocorticoid treatment of the SVMmycH clones increased pSVMmycH expression without inducing endogenous *c-myc* transcripts (lanes e, f, m, and n). Most importantly, dexamethasone induction of DNA synthesis in the SVMmycH clones (Table 2) is caused by induction of *myc* RNA to levels similar to that seen in FGF-stimulated normal 3T3 cells (compare lane f with lane c, lane n with lane o). The SV40mycH1 clone (whose DNA synthesis is not inducible by glucocorticoid) was confirmed to bear elevated levels of *myc* RNA in the absence or presence of dexamethasone (Fig. 3B, lanes c and f).

The rates of *myc* protein synthesis roughly parallel the levels of *myc* RNA in defined medium culture. BALB/c 3T3 and SVMmycH2 cells under several culture conditions were metabolically labeled for 1 h with [35 S]methionine, and total cell extracts were immunoprecipitated with a polyclonal antiserum raised against human *c-myc* protein (provided by R. Eisenman). These immunoprecipitates were analyzed by sodium dodecyl sulfate-gel electrophoresis and fluorography. Dexamethasone induced 67-kilodalton *myc* protein synthesis in SVMmycH2 cells to a level (Fig. 4, lane A) equal to or somewhat higher than that seen in FGF-stimulated 3T3 cells (lane D). The antiserum also bound to several polypeptides with apparent molecular weights of less than 67,000. Whether these additional species represent precursors in posttranslational modification of *myc* protein or are degradation products has not been determined.

We examined the levels of *myc* RNA in SVMmycH2 cells at various times after stimulation with dexamethasone. The

FIG. 4. Metabolically labeled *c-myc* protein in SVMmycH2 cells and normal 3T3 cells. SVMmycH2 and BALB/c 3T3 cells were growth arrested in DMI and fed with DMI with or without dexamethasone or FGF, and after 8 h were refed with DME plus 300 μ Ci of [35 S]methionine per ml. After 1 h, cell lysates were prepared, and 10^7 cpm (trichloroacetic acid precipitable) of each extract were reacted with a rabbit antiserum to *myc* proteins (provided by R. Eisenman). Immunocomplexes were bound to protein A-Sepharose, eluted, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Immunoprecipitates were from extracts of SVMmycH2 cells treated with (lane A) or without (lane B) dexamethasone or from extracts of 3T3 cells treated with (lane D) or without (lane E) FGF. Control normal rabbit serum was reacted with extracts of FGF-induced 3T3 cells and processed in parallel with immunoprecipitates (lane C). Protein molecular weight markers ($\times 10^3$) are indicated to the right, and the arrowhead at the left denotes the 67-kilodalton *c-myc* protein.

FIG. 5. Duration of induced *c-myc* expression in SVMmycH2 cells. DMI-arrested SVMmycH2 cells were refed with DMI containing dexamethasone or FGF, and cytoplasmic RNAs were prepared at several subsequent time points. *c-myc* RNAs were detected by heteroduplex analysis. RNA was prepared from SVMmycH2 cells at 0 h (lane d), 15 h (with dexamethasone) (lane e), 15 h (with FGF) (lane f), 23 h (with dexamethasone) (lane g), 23 h (with FGF) (lane h), and 40 h (with dexamethasone) (lane i). RNA was also from 8-h-old FGF-induced normal BALB/c 3T3 cells (lane j), and lane c shows hybridization mix without cytoplasmic RNA. Lane b, Undigested RNA probe; lanes a and k, ϕ X174 *Hinf*I DNA fragments. Nucleotide lengths of markers are at left, and arrowheads at right mark positions of protected fragments resulting from endogenous (lower) and pSVMmycH (upper) *c-myc* RNAs.

amount of pSVMmycH transcripts persisted at elevated levels for at least 40 h postinduction (Fig. 5). Hence, the suboptimal mitogenic response of SVMmycH2 cells upon pSVMmycH induction cannot be attributed to transient *myc* gene expression.

We performed three experiments to confirm that DNA synthesis that follows dexamethasone induction of pSVMmycH reflects transit of cells from the G1 to the S phase of the cell cycle. One test was to monitor the expression of the histone H4 gene, which is preferentially expressed during the S phase (10). Cytoplasmic RNAs were prepared from cell cultures which had been growth arrested and then maintained for 20 h in DMI with FGF, dexamethasone, or no supplements. Samples were hybridized to a histone H4 gene probe after gel electrophoresis and filter blot hybridization. Induction of pSVMmycH-positive cells with dexamethasone stimulated histone H4 mRNA synthesis, while dexamethasone had no effect on H4 expression in normal BALB/c 3T3 cells (Fig. 6).

We tested whether DNA synthesis induced by dexamethasone in SVMmycH clones is aphidicolin sensitive. S-phase DNA synthesis is predominantly mediated by aphidicolin-sensitive DNA polymerase α , while DNA repair is mediated by aphidicolin-insensitive DNA polymerase β (12, 23). Dexamethasone induction of DNA synthesis in SVMmycH2 cells was inhibited by aphidicolin (Table 2, experiment 4). Aphidicolin does not block expression of *myc* RNA, but does block dexamethasone-induced elevation of histone H4 mRNA levels (data not shown). In fact, aphidicolin actually inhibits DNA synthesis below the basal level of untreated SVMmycH2 cells. Presumably, basal DNA synthesis represents the passage of some cells into the S phase in the absence of glucocorticoid.

FIG. 6. Histone H4 RNA expression in normal and pSVMmycH-transfected BALB/c 3T3 cells. SVMmycH2 cells and normal BALB/c 3T3 cells were DMI arrested and refed with DMI plus FGF, dexamethasone, or no supplements. Cytoplasmic RNAs were prepared 20 h later, and 10 μ g of each RNA was analyzed by filter blot hybridization for histone H4 RNA. RNA was from BALB/c 3T3 cells refed without supplements (lane a), with dexamethasone (lane b), or with FGF (lane c); or from SVMmycH2 cells refed without supplements (lane d), with dexamethasone (lane e), or with FGF (lane f). Arrowhead denotes position of 600-base histone H4 RNA (10).

We also monitored the DNA content in normal and pSVMmycH-positive 3T3 cells at various times after treatment with dexamethasone or FGF. Relative DNA content in individual cells was assayed by staining fixed cell suspensions with the DNA-specific chromophore chromomycin A3 and measuring DNA content by fluorescence intensity in a fluorescence-activated cell sorter. Figure 7 shows fluorescence profiles of cell populations. Growth-arrested normal 3T3 cells had almost exclusively a G1 DNA content. FGF induced a somewhat synchronous transit of cells through the S phase and into G2 over the ensuing 23 h, while dexamethasone did not induce cell transit. In this experiment, between 85 and 90% of SVMmycH2 cells arrested with DMI had a G1 content of DNA. Dexamethasone treatment increased to 30% the fraction of cells with a DNA content greater than that in G1, and most of these cells had reached G2 by 23 h postinduction. The failure of all SVMmycH2 cells to cycle upon glucocorticoid administration cannot be attributed to reduced cell viability since a large fraction of these H2 cells could cycle upon treatment with FGF.

DISCUSSION

We have shown that the induction of *c-myc* gene expression in 3T3 cells deprived of PDGF and FGF induces DNA synthesis and that a fraction of the induced cells traverse the S phase. This cellular response occurs at physiological levels of *myc* gene expression. However, we observed that DNA synthesis after *myc* gene (pSVMmycH) induction is not as pronounced as that after FGF induction. Furthermore, induction of pSVMmycH did not appreciably stimulate cell growth. These results could reflect a failure of the *c-myc* gene product to provide all the signals and substrates for cell growth. FGF or PDGF may induce other essential gene functions before or in parallel with *c-myc* induction. For example, growth factors induce rapid and transient expression of the *c-fos* proto-oncogene (8), and it has been reported that *c-fos* expression is required for fibroblast proliferation (11, 17). The *c-fos* function may act in concert with *c-myc* and, perhaps, other cellular constituents to efficiently mediate cell cycling.

Our data for the role of *c-myc* expression in fibroblast growth differ from those of conceptually similar studies conducted on BALB/c 3T3 cells cultured in PDGF-poor plasma (2). The induction of a transfected *myc* gene in plasma-arrested cells stimulated DNA synthesis and induced

FIG. 7. DNA content in SVMmycH2 and normal BALB/c 3T3 cells after dexamethasone (Dex) or FGF induction. The SVMmycH2 clone (abbreviated H2) and normal BALB/c 3T3 cells were DMI arrested and refed with DMI containing dexamethasone or FGF. Cells were harvested at 0, 15, and 23 h, fixed, and stained with chromomycin A3 and analyzed for relative fluorescence as a measure of DNA content. A total of 20,000 cells were analyzed per sample, and the data are displayed as histograms with relative fluorescence on the horizontal axes and cell number on the vertical axes. For each sample, cells with greater than the G1 DNA content lie to the right of the arrowhead marking channel 80. The percentages of cells in this fraction are: SVMmycH2 cells, 0 h, 14%; dexamethasone, 15 h, 30%; dexamethasone, 23 h, 27%; FGF, 15 h, 70%; FGF, 23 h, 50%; BALB/c 3T3 cells, 0 h, 7%; dexamethasone, 15 h, 7%; dexamethasone, 23 h, 7%; FGF, 15 h, 65%; FGF, 23 h, 73%.

substantial cell proliferation, leading to the conclusion that *c-myc* expression eliminated the cellular requirement for PDGF. It is possible that plasma contains one or more factors which complement *c-myc* expression to mimic PDGF induction of cell growth. Alternatively, *c-myc* expression could lower, but not eliminate, the PDGF requirement of 3T3 cells. Low levels of PDGF may be found in plasma preparations, as evidenced by proliferation of a fraction of

untransfected BALB/c 3T3 cells in plasma-supplemented medium (2).

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