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Posttranscriptional Regulation of Cellular Gene Expression by the c-*myc* Oncogene

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The c-*myc* oncogene has been implicated in the development of many different cancers, yet the mechanism by which the c-*myc* protein alters cellular growth control has proven elusive. We used a cDNA hybridization difference assay to isolate two genes, *mr1* and *mr2*, that were constitutively expressed (i.e., deregulated) in rodent fibroblast cell lines immortalized by transfection of a viral promoter-linked c-*myc* gene. Both cDNAs were serum inducible in quiescent G_0 fibroblasts, suggesting that they are functionally related to cellular proliferative processes. Although there were significant differences in cytoplasmic mRNA levels between *myc*-immortalized and control cells, the rates of transcription and mRNA turnover of both genes were similar, suggesting that c-*myc* regulates *mr1* and *mr2* expression by some nuclear posttranscriptional mechanism. *mr1* was also rapidly (within 2 h) and specifically induced by dexamethasone in BALB/c cell lines expressing a mouse mammary tumor virus long terminal repeat-driven *myc* gene, under conditions where other growth factor-inducible genes were unaffected. A frameshift mutation in the mouse mammary tumor virus *myc* gene destroyed the dexamethasone stimulation of *mr1*, indicating that c-*myc* protein is required for the effect. As in the *myc*-immortalized cells, the induction of *mr1* by c-*myc* occurred without detectable changes in *mr1* transcription or cytoplasmic mRNA stability, implicating regulation, either direct or indirect, through a nuclear posttranscriptional mechanism. These results provide evidence that c-*myc* can rapidly modulate cellular gene expression and suggest that c-*myc* may function in gene regulation at the level of RNA export, splicing, or nuclear RNA turnover.

The c-*myc* gene encodes a nuclear protein whose expression is closely associated with the proliferative state of many mesenchymal cells. It is activated during oncogenesis by a variety of mechanisms and appears to play a central role in the etiology of many tumors. The levels of c-*myc* are controlled by the action of growth factors, and constitutive expression of the gene in some fibroblast systems partially relieves dependence on these factors for entrance into the cell cycle (1, 5, 41, 43; for reviews, see references 3, 10, and 22). Deregulated c-*myc* expression can also block the differentiation of several cell types (10). These observations suggest that c-*myc* plays a central role in the regulatory networks that control cellular proliferation and differentiation. An understanding of the molecular function of c-*myc* could be greatly facilitated by the study of genetic targets that are relevant to cellular transformation processes.

We approached this problem by isolating genes that were protential targets for c-*myc* activity, i.e., genes whose expression was deregulated in c-*myc*-immortalized rodent fibroblasts. Using a cDNA hybridization difference assay, we isolated two cDNAs, *mr1* and *mr2*, which represent cellular mRNAs constitutively expressed in *myc*-immortalized cells but not in cells immortalized by adenovirus E1a or growth-arrested normal cells. These same genes were rapidly induced in quiescent established rat fibroblast lines in response to serum growth factors. Induction of both *mr1* and *mr2* by c-*myc* did not alter the transcription or mRNA turnover of these genes, inferring that a posttranscriptional mechanism operating in the nucleus is responsible for the deregulation. This work provides molecular evidence that c-*myc* regulates cellular gene expression and suggests a novel gene-regulatory function for the c-*myc* protein.

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MATERIALS AND METHODS

Construction of cDNA library. A cDNA library was prepared from BRK nH*myc*3 poly(A)$^+$ RNA selected twice on oligo(dT) cellulose (Collaborative Research, Inc.) by using the λgt10 vector and the modified Okayama-Berg method (15), except that Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) and 50 ng of actinomycin D per ml were used in the first-strand reaction. The original library contained about 1.7×10^5 recombinants before amplification.

cDNA hybridization difference screening. Duplicate nitrocellulose blots from phage plates containing 500 PFU were prepared as described previously (32). Baked filters were washed overnight at 37°C in 10 ml (per filter) of 10 mM Tris chloride (pH 7.8)–5 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–50 μg of proteinase K per ml, gently rinsed in a large volume of boiling water for 5 min, and then washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C for 30 min. Prehybridization was performed in large ZipLock bags (Dow Chemical Co.) on a rocking platform (2 to 3 oscillations per min; Bellco Glass, Inc.) at 37°C for 8 to 20 h in 5 ml (per filter) of 6× SSC–50 mM NaH_2PO_4–5 mM sodium pyrophosphate–5× Denhardt solution–100 μg of sheared salmon sperm DNA per ml–5 μg of sheared *Escherichia coli* DNA per ml–50% formamide. Hybridization proceeded for 3 to 4 days on the rocking platform in the same buffer (3 to 4 ml per filter) in fresh solution containing the labeled cDNA probe. Filters were washed once at 23°C in 2× SSC–0.1% SDS and three times at 65°C in 0.2× SSC–0.1% SDS and then autoradiographed for up to 10 days. Probes were prepared from 3 to 5 μg of poly(A)$^+$ RNA in a first-strand reaction with 1,000 U of Moloney murine leukemia virus reverse transcriptase and 50 μCi of [α-^{32}P]CTP; no cold CTP was included during the first 30 min of the 60-min reaction. The probe was purified by

chromatography through G-100 Sephadex (Sigma Chemical Co.), boiled for 5 min, and passed through a 0.45-μm-pore-size nitrocellulose acetate syringe filter (Gilman).

Plasmid constructions. All cDNA inserts were subcloned into either pUC19 or the pBS^+ vector (Stratagene) by standard protocols (32). The Tn5*neo* gene, driven by an internal β-actin promoter, was inserted into the c-*myc* expression vector pEVX-XH*myc* (2) to generate a recombinant retroviral construct, *myc*XH-S7. This construct was transfected into ψ2 cells (33) and the helper-free supernatant used for infections.

A three-exon recombinant mouse mammary tumor virus (MMTV)-*myc* gene, pXhR/*his*, was generated by fusing an *Xho*I-*Eco*RI human genomic DNA fragment containing the normal human c-*myc* gene to a cloned MMTV promoter. By use of this construct, a frameshift mutation was generated in the human c-*myc* gene to produce a disfunctional, dexamethasone-inducible gene. This was accomplished by digestion to completion of the unique *Bst*EII site in c-*myc* exon 2, blunt-end repair of the overhanging 5' termini with Klenow polymerase, and self-ligation of the blunt-ended product. The predicted mutation in this plasmid, pXhR/Bst, was verified by dideoxy sequencing a convenient subclone of the appropriate region. Both these plasmids contain the dominant selectable marker SV_2*his*, which confers resistance to the animal cell toxin histidinol (I. Lemischka, personal communication).

Cell lines and experimental culture conditions. The BRK cell lines used have been described (21; A. Kelekar, Ph.D. thesis, Princeton University, Princeton, N.J., 1987). Briefly, secondary BRK cultures were cotransfected with pRSV*neo* and *onc* expression vectors by standard protocols (12), and G418-resistant colonies were expanded into mass culture. The nH*myc*1 and nH*myc*3 cell lines were generated with pH*myc*LTR, the n*ras*3 line was generated with pEJ (21), and the E1a2 and nE1a2 lines were generated with p1A (20). E1a2-*neo* and E1a2-XH were generated by transfection of the E1a2 parent with pRSV*neo* or with pRSV*neo* and the c-*myc* expression vector pEVX-*myc*XH. The BRK E1a-*myc* cell line was created by infection of the BRK E1a2 parent with the *myc*XH-S7 retrovirus described above. Ring-cloned G418-resistant colonies were expanded into mass culture, and one resultant cell line, designated E1a-*myc*, was used for this study. The BALB/c mouse fibroblast lines 9E and 4A were obtained from the laboratory of C. Stiles. 9E was created by cotransfection of BALB/c 3T3 cells with an MMTV long terminal repeat-driven mouse c-*myc* gene and HSV*neo*, and 4A was generated as a control line by transfection with HSV*neo* alone (1). The REF52 cell line, provided by A. Levine (31), is a spontaneously immortalized fibroblastoid line which was cloned from a rat embryo fibroblast preparation at passage number 52.

All cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 4 mM glutamine (Sigma) and 10% fetal calf serum (GIBCO Laboratories). Subconfluent cultures were approximately 50% confluent and were fed 24 h before harvesting. Confluent cultures were density arrested for 1 or 2 days in medium that had been depleted by the cells for 3 to 5 days. For the BALB/c 3T3 experiments, quiescent cells were typically produced by density arrest 2 days in DMEM containing 10% fetal calf serum and then incubation for 4 h in DMEM containing 0.3% fetal calf serum. For one experiment (see Fig. 6), 4A and 9E cells were made quiescent by density arrest for 2 days followed by an overnight incubation in DMEM containing 5% platelet-poor plasma (PPP). For growth factor stimulations, platelet extract containing crude platelet-derived growth factor (PDGF; a gift of C. Stiles [37]) was added to a final concentration of 200 U/ml, and dexamethasone (Sigma) was added to 1 μM. For RNA half-life studies, the transcription inhibitor actinomycin D was added directly to culture media to a final concentration of 5 μg/ml.

Cell transfections. BALB/c mouse fibroblasts, clone A31 (ATCC), were transfected with recombinant MMTV-*myc* plasmids by the calcium phosphate precipitate method (17). One day after transfection, cells were split 1:5; after an additional day, the *his* marker was selected for by the addition of histidinol (Sigma) to the medium to a final concentration of 5 μg/ml. After 2 weeks of selection, drug-resistant colonies were ring cloned and expanded into mass culture.

RNA analysis. Total cytoplasmic RNA was prepared as described previously (19). For Northern analysis, 8 μg of RNA per lane was fractionated on 0.8% agarose–2.2 M formaldehyde gels and transferred to GeneScreen membranes (Dupont, NEN Research Products). Blots were processed and hybridized (6) with [α-^{32}P]DNA probes labeled by the random primer method (Pharmacia Fine Chemicals). Filters were washed once for 15 min at room temperature in 2× SSC–0.1% SDS and then twice for 10 min at 60°C in 0.2× SSC–0.1% SDS. Before rehybridization, blots were stripped of old probes by washing twice for 15 min in 0.02× SSC–0.1% SDS heated to 100°C. For autoradiography, blots were exposed to Kodak XAR-5 film with intensifying screens (DuPont). All autoradiographs were subjected to densitometer (Bio-Rad Laboratories) scanning to equalize the RNA loaded in each lane and determine the fold inductions that were observed between lanes.

Nuclear run-ons. Preparation of nuclei and nuclear run-on reactions were performed essentially as described previously (29) with double-stranded plasmid clones as hybridization targets.

Examination of cytoplasmic RNA stability. Cells were treated for various times with the transcription inhibitor actinomycin D (Sigma) at a final concentration of 5 μg/ml. The decay rate of *mrl* mRNA was examined by Northern analysis of equivalent amounts of total cytoplasmic RNA prepared from treated cells. Autoradiographs were subjected to densitometer scanning analysis, and relative band intensity readings were plotted as a function of the time of transcription inhibition by actinomycin D. *mrl* RNA half-life was determined from these autoradiographs by a least-squares linear regression calculation of the plotted data.

RESULTS

Basis for a differential cDNA screen with E1a- and c-*myc*-immortalized BRK fibroblasts. The identification of cellular genes specifically regulated by an oncogene can provide important tools for understanding the alterations in growth imposed by that oncogene. However, a major difficulty in designing an appropriate screening protocol for genes whose expression correlates with c-*myc* is the close linkage between c-*myc* expression and entry into the cell cycle. It was of particular interest to distinguish those cellular genes responsive to c-*myc* from among the larger class of genes that are induced during the transition from quiescence to growth. Toward this end, we noted that in BRK lines created by immortalization with the adenovirus type 5 E1a gene the c-*myc* gene is expressed at low levels, even though the cells are actively dividing in the presence of serum growth factors (21). Because c-*myc* expression and entrance into the S

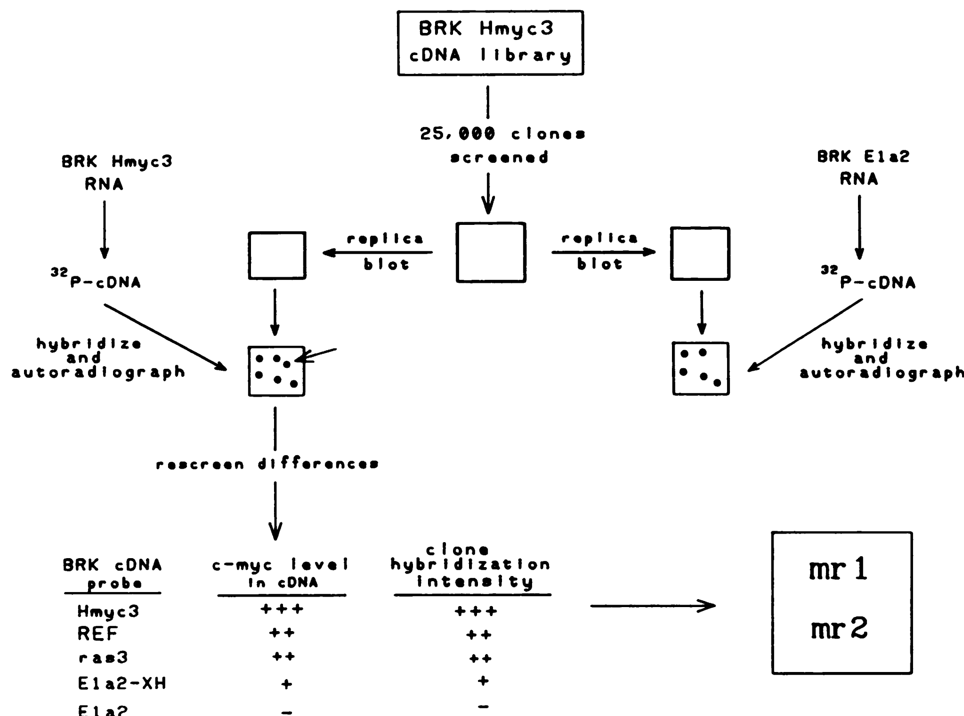

FIG. 1. Strategy for the cDNA hybridization difference screen. A cDNA library was consrtucted from BRK nH*myc*3 fibroblast poly(A)$^+$ RNA in λgt10 and screened initially with cDNA probes expressing relatively higher (BRK/nH*myc*3, BRK/E1a2-XH) or lower (BRK/E1a2) levels of c-*myc* RNA. Clones giving a positive difference were picked and rescreened with cDNA probes with varied c-*myc* RNA levels as shown. See the text for details.

phase was uncoupled in E1a-immortalized BRK cells, we elected to compare cDNA populations in E1a- and c-*myc*-immortalized lines in an effort to isolate a set of genes whose expression correlated with c-*myc* but not with growth per se. Genes in such a set might be relevant to c-*myc* immortalization (i.e., their expression may be modulated by c-*myc* activity) and might represent candidate targets for c-*myc* activity. The screening protocol made two major assumptions: first, that the c-*myc* oncogene would modulate gene expression monitorable at the level of cytoplasmic RNA; and second, that there would exist a set of genes modulated by c-*myc* activity that were not also modulated by E1a. A further expectation was that a gene responsive to c-*myc* would also be modulated by serum growth factors and would be deregulated in c-*myc*-immortalized cells.

Isolation of cellular genes whose expression correlates with c-*myc* in immortalized BRK fibroblast lines. The strategy for the cDNA difference screening with multiple cDNA probes on replica phage blots is outlined in Fig. 1. A cDNA library was constructed with mRNA from BRK nH*myc*3 (21), a line of BRK cells immortalized by long terminal repeat-H*myc* (20) that expresses three to four times the level of c-*myc* RNA found in HeLa cells (unpublished observations). Initially, duplicate filters containing approximately 9,000 plaques were screened with cDNA probes from nH*myc*3 (homologous) and E1a2 cDNA probes. In the second experiment, 16,000 plaques were screened with cDNA probes from E1a2 and E1a2-XH, a derivative of E1a2 generated by transfection with pEVX-*myc*XH (a c-*myc* expression vector) and pRSV*neo*. This second screen was expected to be more specific for those genes responding to increased c-*myc* levels in an E1a-immortalized cellular background. The cDNA probes were derived from cytoplasmic poly(A)$^+$ RNA in all cases.

From the initial screens, about 380 plaques showed differences and were rescreened with cDNA probes from the following lines: nH*myc*3, E1a2, E1a2-XH (a line immortalized by E1a and secondarily transfected with a c-*myc* expression plasmid), n*ras*3 (a line immortalized by the H-*ras*$^{\mathrm{Val\text{-}19}}$ oncogene), and from cultured primary rat embryo fibroblasts (REF cells). These probes were chosen both for their heterogeneity and for the various levels of c-*myc* RNA represented in the cDNA. The endogenous c-*myc* gene is deregulated in cells immortalized by low levels of the H-*ras* oncogene, so this probe was chosen to select for genes whose expression correlated with c-*myc* in another type of immortalized cell line. The REF probe was included to select for those genes that correlated with c-*myc* in nonimmortalized primary cells. We wished to monitor differences that were dosage dependent with c-*myc* and that could be manifested in many cellular backgrounds.

We found that 20 of the original 380 differences correlated positively with relative c-*myc* abundance in all five probes. Cross-hybridization between the clones revealed that eight or nine different sequences were represented. From these phage, cDNA inserts were used to probe blots of both DNA and RNA from various BRK lines. Two of the clones, *mr1* and *mr2*, represented cellular genes that are conserved in evolution (data not shown) and exhibited the differential expression initially screened for; i.e., they hybridized to specific RNA(s) which are expressed in confluent, growth-arrested c-*myc*-immortalized cells but not in E1a-immortalized cells (Fig. 2). The relative c-*myc* RNA levels varied greatly in these cells, as observed previously (21; Kelekar, Ph.D. thesis); Hybridization with β-actin was used to show the approximate amount of RNA loaded in each lane of the gel. We observed two RNAs hybridizing to *mr1*: a major species of 3.5 kilobases (kb) and a minor species of 2.3 kb.

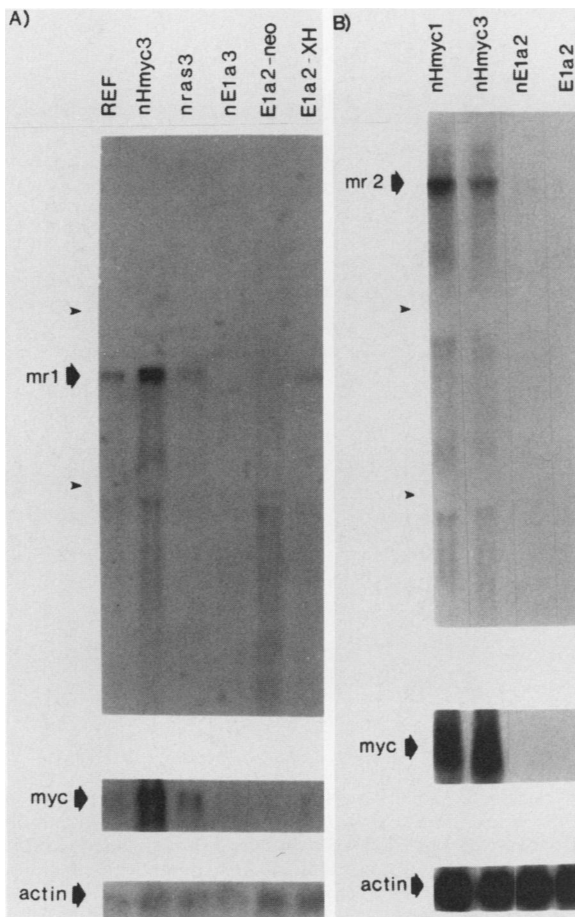

FIG. 2. *mr1* and *mr2* cDNAs are expressed in confluent BRK cell lines created by immortalization with c-*myc* but not adenovirus E1a. Total cytoplasmic RNA from each cell line was examined by Northern analysis with the cDNA inserts, c-*myc* mouse exon 2 (*Xba*I-*Sac*I genomic fragment), and β-actin as probes. All cells were confluent when RNA was prepared. Arrowheads indicate the position of the 18S and 28S rRNA bands on the Northern blots. (A) Northern analysis of *mr1* expression in cell lines from which the original cDNA probes were derived for the difference screen; 10 μg of RNA per lane was analyzed. (B) Northern analysis of *mr2* expression in two c-*myc*- and two E1a-immortalized cell lines; 8 μg of RNA per lane was examined.

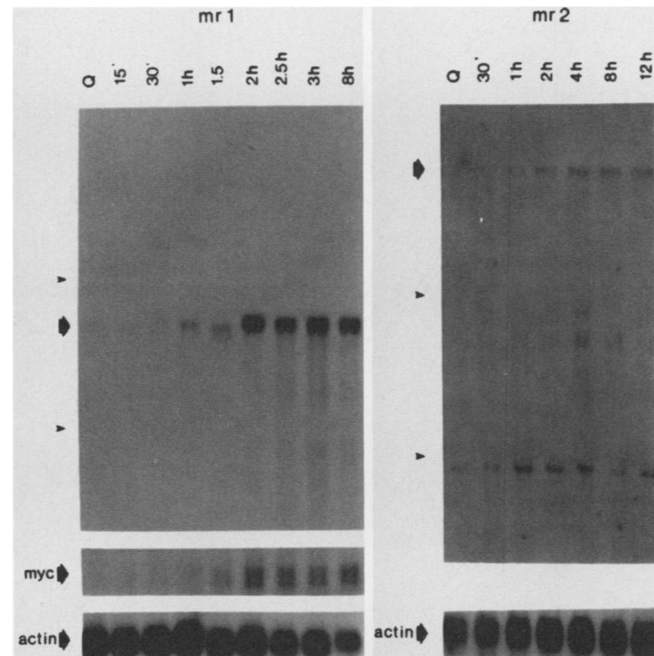

FIG. 3. Serum growth factors induce expression of *mr1* and *mr2* RNA in quiescent established rat fibroblasts. Quiescent, density-arrested REF52 fibroblast monolayers were stimulated by the addition of fresh medium containing 10% fetal calf serum. Total cytoplasmic RNA was prepared at the times indicated, and 8 μg per lane was examined by Northern analysis. The slight decrease in size of *mr1* RNA apparent at 1.5 h is due to a small change in the RNA mobility in this lane of the gel. c-*myc* and β-actin levels were examined as above on blots stripped of the initial cDNA probes.

For *mr2*, a single ~12-kb species was found. (On some gel blots, this large *mr2* transcript could be resolved into two closely sized bands; in addition, there were minor species of ~1.8 and 4.0 to 4.5 kb which weakly cross-hybridized on some blots). The cDNA inserts from *mr1* and *mr2* were 1.3 and 2.9 kb, respectively, and contained information from the 3′ ends of the respective RNAs (data not shown).

***mr1* and *mr2* rapidly induced by serum growth factors in established fibroblasts.** We predicted that *mr1* and *mr2* genes would be modulated with the growth state, in parallel with c-*myc*, in established cells that harbor neither E1a nor deregulated c-*myc* genes. To test this, Northern analysis of RNA from quiescent and serum-stimulated REF52 fibroblasts was performed (Fig. 3). Both genes were expressed at low levels in quiescent cells and induced by serum within 2 h of addition. Densitometric scanning of the autoradiographs, normalized to the actin controls, indicated that *mr1* RNA levels increased ~20-fold at 2 h and that the large *mr2* transcript increased 2- to 3-fold. Induction of c-*myc* occurred with approximately the same kinetics as did both *mr1* and *mr2* (Fig. 3). These results strongly suggest that these genes are functionally involved in cellular proliferative responses, since their expression is activated by serum growth factors. Furthermore, these observations are consistent with the hypothesis that *mr1* and *mr2* are responsive to c-*myc* in fibroblast cell lines, since the activation of their expression did not precede c-*myc* stimulation.

***mr1* and *mr2* deregulated in c-*myc*-immortalized BRK cell lines.** We predicted that expression of both *mr1* and *mr2* would be deregulated in c-*myc*-immortalized BRK cells by comparison to E1aBRKs and normal REF52 fibroblasts. To test this, steady-state mRNA levels were compared between subconfluent, growing cells and confluent, density-arrested cells. At confluence, *mr1* was expressed at very low levels in E1aBRK fibroblasts; however, *mr1* was constitutively expressed in confluent *myc*BRKs at a level similar to that found in subconfluent cell cultures (Fig. 4A). *mr2* was also expressed at similar levels in subconfluent *myc*BRKs and E1aBRKs; in confluent cultures, however, levels of the large *mr2* transcript actually increased in *myc*BRKs (Fig. 4B). Similar to *mr1*, this mRNA was down-regulated at confluence in the E1a-immortalized cells.

To further explore the response of *mr1* and *mr2* to c-*myc* levels, we introduced a deregulated c-*myc* gene into E1aBRKs to determine whether this would result in the same constitutive expression observed in the c-*myc*-immortalized lines. In E1a-*myc*, both *mr1* and *mr2* were constitutively expressed at confluence, in contrast to the E1a2 parental line (Fig. 4C). The regulation of *mr1* in E1a-

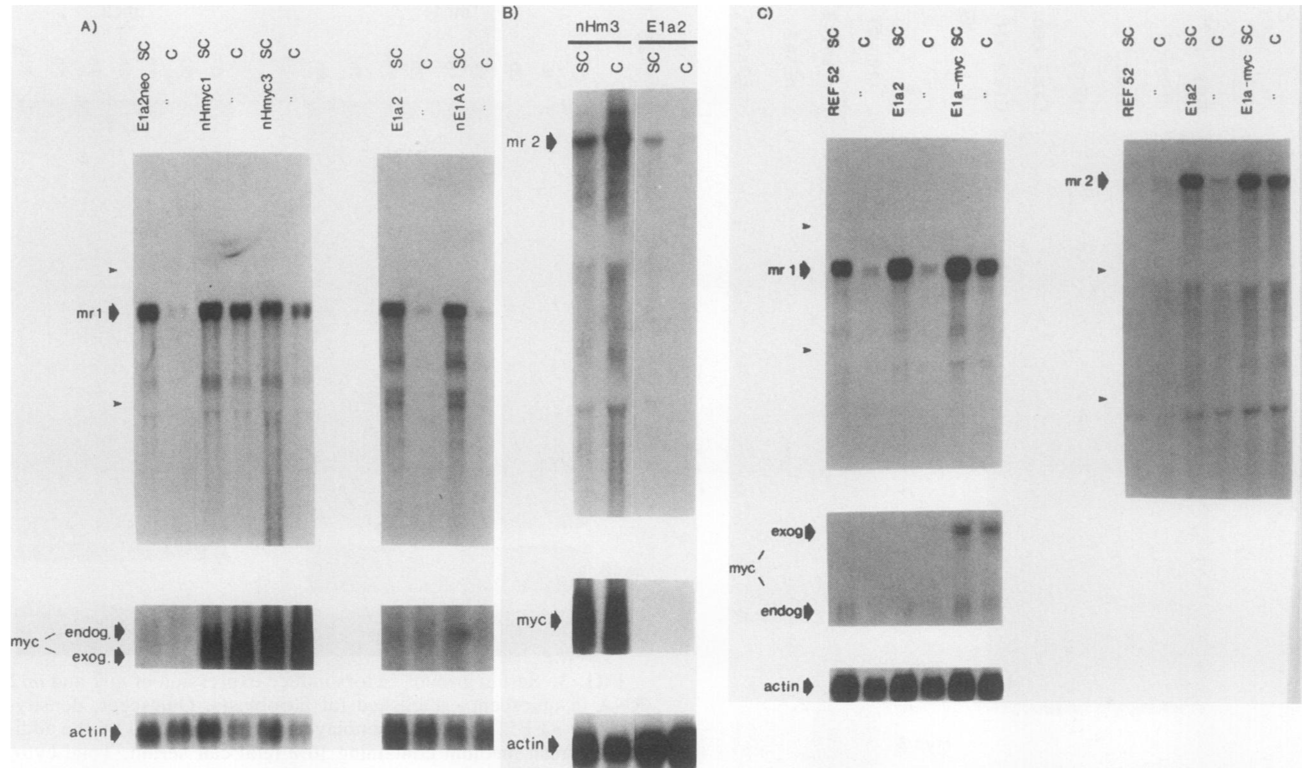

FIG. 4. *mr1* and *mr2* are deregulated in BRK cells constitutively expressing c-*myc*. Total cytoplasmic RNA from subconfluent and confluent cells was isolated and analyzed by Northern blotting (8 μg of RNA per lane). (A) RNAs from two c-*myc*-immortalized and three E1a-immortalized BRK lines were analyzed for *mr1* deregulation. (B) RNAs from H*myc*3 and E1a2 were examined for deregulation of mr2. The lower panels showing c-*myc* hybridization in A and B were derived from separate blots with equivalent amounts of RNA. Each blot was stripped and rehybridized with β-actin. (C) RNAs from REF52, BRK E1a2, and BRK E1a-*myc* were analyzed. The blot was successively hybridized to *mr2* cDNA, c-*myc* exon 2, *mr1* cDNA, and β-actin.

immortalized cells paralleled that in growing or growth-arrested REF52 fibroblasts (Fig. 4C), indicating that the down-regulation in E1a-immortalized cells is probably not due to E1a-induced repression (4, 46). With regard to *mr2*, neither cycling nor arrested REF52 cells expressed the gene at a level comparable to that of BRK fibroblasts (Fig. 4C). Since cycling cells do not enter the G_0 phase (35), and because *mr2* mRNAs are induced by serum in quiescent, growth-arrested cells (Fig. 3), expression of *mr2* may be activated in REF52 fibroblasts by serum growth factors only during the G_0-G_1 transition of the cell cycle.

c-*myc* regulation of *mr1* and *mr2* at some posttranscriptional level not including mRNA stability. To determine whether transcription of *mr1* and *mr2* was modulated by c-*myc* expression, we compared nuclear run-on transcription in both subconfluent and confluent cultures of *myc*BRKs and E1aBRKs. In addition to *mr1* and *mr2*, we monitored transcription of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is not growth regulated and serves as a standard for comparison between blots. Figure 5A shows results which parallel the Northern analysis in Fig. 4A and B, whereas Fig. 5B shows E1a2 and E1a-*myc* transcription corresponding to the Northern analysis shown in Fig. 4C.

Relatively small differences were observed in *mr1* transcription between E1aBRK and *myc*BRK lines by comparison with the significant variations in their steady-state cytoplasmic RNA levels (Fig. 5A). In confluent cells, *mr1*

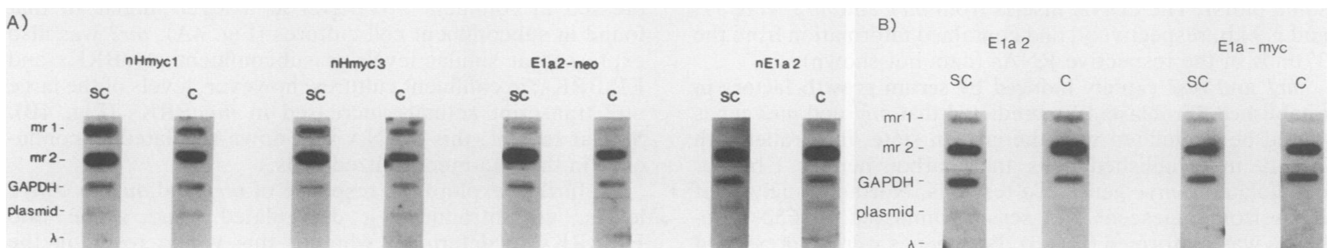

FIG. 5. Nuclear run-on transcription in BRK cells constitutively expressing c-*myc*. Nuclei from subconfluent and confluent cells were processed and analyzed as described previously (29). DNA targets for hybridization were 8 μg of linearized plasmid containing *mr1* cDNA, *mr2* cDNA, avian GAPDH, plasmid vector (pUC19), and *Hae*III-digested lambda bacteriophage DNA (λ). (A) Run-on transcription from two c-*myc*- and two E1a-immortalized lines is shown. (B) Run-on transcription from E1a2 and E1a-*myc* is shown.

transcription was three- to fourfold higher in *myc*BRKs than in E1aBRKs. This increase cannot account for the 20-fold difference in cytoplasmic mRNA observed between lines. It might be argued that this is correlated with higher c-*myc* expression in these cell lines and may represent a transcriptional activation of *mr1* by c-*myc*. However, *mr1* transcription in subconfluent *myc*BRKs decreased as the cells reached confluence (Fig. 5A), whereas the cytoplasmic mRNA level was unchanged (Fig. 4A). The converse situation was observed in E1aBRKs; here, *mr1* was transcribed at the same rate in subconfluent and confluent cells (Fig. 5A), even though it was down-regulated in the cytoplasm at confluence (Fig. 4A). Analysis of the E1a-*myc* line indicates that constitutive expression of c-*myc* in an E1a-immortalized background had little effect on *mr1* transcription (Fig. 5B) with the same conditions under which c-*myc* deregulated *mr1* in these cells at the level of cytoplasmic RNA (Fig. 4C). Since it appears that constitutive c-*myc* expression does not result in constitutive *mr1* transcription, and transcription was not elevated in E1aBRK cells after introduction of a constitutive c-*myc* gene (Fig. 5B), we suggest that the slightly higher level of *mr1* transcription in *myc*BRKs may be due to some indirect effect of c-*myc* immortalization.

Transcription of *mr2* was quite high in both subconfluent and confluent cultures of all cell lines (Fig. 5). The strong level of hybridization observed in these run-on experiments was not due to the presence of repetitive sequences in the *mr2* target, since the same cDNA showed single-copy abundance when hybridized to genomic Southern blots. Transcriptional activity of *mr2* was quite similar in both c-*myc*- and E1a-immortalized BRKs (Fig. 5A) under all conditions. This is in stark contrast to the great differences in cytoplasmic *mr2* RNA abundance (Fig. 4B). Consistent with these results, *mr2* transcription was virtually identical in E1a-*myc* and E1a2 cells (Fig. 5B) under conditions which manifested ~10-fold differences in cytoplasmic RNA (Fig. 4C).

In contrast to *mr1* and *mr2*, transcription of a PDGF-inducible gene, JE, quantitatively paralleled the levels of cytoplasmic RNA expressed in these cells, being slightly higher in c-*myc*-immortalized BRKs (21; data not shown). We also examined hsp70 transcription, since it has been reported that c-*myc* increases transcription from the hsp70 promoter in transient 3T3 transfection assays (18). However, we observed no difference in hsp70 transcription in cells immortalized by c-*myc* or E1a (data not shown). The low level of background hybridization to pUC19 in some hybridizations is probably due to run-on transcription into plasmid-derived sequences present in these cell lines.

The run-on assays above monitor the net transcription through the genes tested. However, an alternative hypothesis is that c-*myc* might modulate transcription elongation rather than initiation, even though the cloned plasmid targets used for hybridization contain partial *mr1* and *mr2* cDNAs with only 3′ information. Nevertheless, we tested this directly by using 5′ and 3′ subclones of a full-length murine *mr1* cDNA (Prendergast et al., manuscript in preparation) as run-on hybridization targets. There were no observable differences in the level of run-on transcription with these targets under any conditions in either E1a or E1a-*myc* cells (data not shown).

Since it was possible that the steady-state increase in *mr1* and *mr2* mRNA levels in *myc*BRKs was due to cytoplasmic RNA stabilization, we examined the message half-life of these genes. Both *mr1* and *mr2* mRNAs were fairly stable, with half-lives of ~2 to 3 and ~5 h, respectively; most significantly, though, stability of these RNAs did not vary in

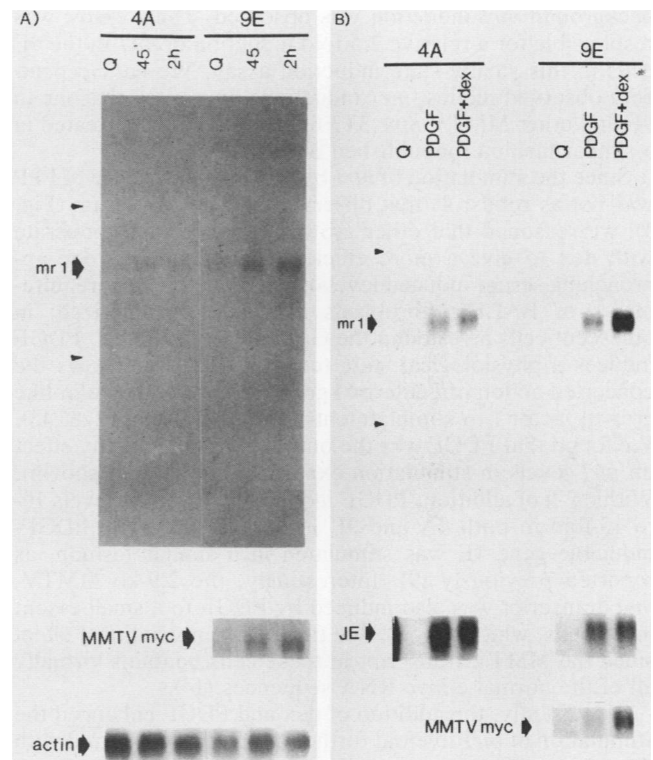

FIG. 6. *mr1* is rapidly induced by dexamethasone in a MMTV-*myc* BALB/c fibroblast line but not in the control line. (A) BALB/c 4A and 9E cells were made quiescent in 5% PPP. RNA was prepared (Q) or dex was added to a final concentration of 1 μM and RNA was prepared at the indicated times after the addition of dex. Northern blots of the RNA were hybridized successively to probes for *mr1* cDNA, mouse c-*myc* exon 2 (*Xba*I-*Sac*I genomic fragment), and β-actin. (B) BALB/c 4A and 9E cells were made quiescent in DMEM containing 0.3% fetal calf serum. RNA was prepared (Q), or the cells were stimulated for 2 h with 200 U of crude PDGF (platelet extract) per ml with or without 1 μM dex. Northern blots were successively hybridized to *mr1* cDNA, mouse c-*myc* exon 2, and JE cDNA (9). Arrowheads indicate the positions of the 18S and 28S rRNA bands on the blots.

response to deregulated c-*myc* expression (data not shown). We conclude that regulation of *mr1* and *mr2* by c-*myc* in BRK cells is not due to an indirect stabilization of cytoplasmic mRNA. Taken together with the run-on data presented above, the results suggest that both *mr1* and *mr2* are deregulated by c-*myc*, either directly or indirectly, at some posttranscriptional level other than mRNA stability.

Specific regulation of *mr1* by dexamethasone in BALB/c MMTV-*myc* 3T3 fibroblasts. To study the interaction of the *mr1* gene and c-*myc* more directly, we examined the specificity and rapidity with which c-*myc* regulated these genes by using a stable cell induction assay with two BALB/c fibroblast lines, 9E and 4A. Stimulation of 9E cells with the steroid hormone dexamethasone (dex) results in a rapid and specific induction of c-*myc* mRNA in the cytoplasm (1).

The addition of dex to 9E cells quiescent in 5% PPP resulted in a 3-fold steady-state increase in cytoplasmic c-*myc* RNA within 2 h, along with a modest but reproducible 3- to 3.5-fold induction of *mr1* (Fig. 6A). Confluent BALB/c 3T3 cells incubated in PPP are translationally competent but mitotically inactive (8) because the PPP medium supplement lacks platelet-derived growth factor (PDGF), a critical mitogen for these cells (45). In the 4A control line, a ~0.7-fold

background *mrl* induction was observed. Thus, c-*myc* was responsible for a relative 2.5-fold induction of *mrl* in the 9E cells in this steady-state induction assay. We have repeatedly observed modest *mrl* inductions by dexamethasone in 9E and other MMTV-*myc* 3T3 fibroblast cell lines treated in a similar fashion (unpublished observations).

Since the stimulation of *mrl* by dex in the presence of PPP was not as robust as that observed with whole serum (Fig. 3), we reasoned that other growth factors might cooperate with dex to give a more efficient induction (i.e., one approaching serum-induced levels). The growth factor requirements of BALB/c fibroblasts are well characterized: in quiescent cells arrested in the G_0 phase of cell cycle, PDGF induces a physiological state (competence) that allows the concerted action of epidermal growth factor and insulin-like growth factor I to stimulate entry into the S phase (28, 45). We found that PDGF was the only factor that had any effect on *mrl* levels in stimulation experiments (data not shown). Within 2 h of addition, PDGF induced *mrl* mRNA levels 10- to 15-fold in both 4A and 9E lines (Fig. 6B). The PDGF-inducible gene JE was stimulated in a similar fashion, as reported previously (9). Interestingly, the 2.9-kb MMTV-*myc* transcript was also induced by PDGF to a small extent in 9E cells, which may be due to a posttranscriptional effect since the MMTV transcript in these cells contains virtually all of the normal c-*myc* RNA sequences (14).

Significantly, the addition of dex and PDGF enhanced the stimulation of *mrl* fivefold further in 9E cells compared with the 4A controls when equalized to JE levels (Fig. 6B). A fourfold increase in MMTV-*myc* RNA levels in 9E was also observed during the 2-h treatment. The augmentation of steady-state RNA levels by dex was specific to *mrl*, since the addition of the steroid had no effect on either JE or three other PDGF- and/or serum-inducible genes, KC (9), p53 (38), and hsp70 (47), up to 6 h after stimulation with PDGF (data not shown).

Frameshift mutations in the exogeneous MMTV-*myc* gene destroy the ability of dexamethasone to regulate *mrl*. To control for the presence of the MMTV promoter in the 9E BALB/c fibroblasts and to define the requirement of c-*myc* protein synthesis for *mrl* regulation, we created BALB/c 3T3 lines containing either normal or frameshifted protein-coding information in the exogeneous MMTV-*myc* gene. The pXhR/*his* plasmid carries a three-exon human c-*myc* gene fused to the MMTV promoter (Fig. 7A), similar to the mouse c-*myc* construct expressed in 9E cells (1). The expected size of the MMTV-*myc* RNA was ~3.0 kb; this transcript was easily resolved from the endogeneous c-*myc* RNA on 0.8% agarose Northern gels. The pXhR/Bst plasmid was identical to its parent, pXhR/*his*, except that it harbored a frameshift mutation at the *Bst*EII site in the human c-*myc* second exon. The frameshift generated a termination codon just after amino acid 104 in the normal c-*myc* protein-coding region (Fig. 7A).

In a cell line expressing the MMTV-*myc* frameshift mutation (Bst2), steady-state *mrl* RNA levels were significantly increased by a 2-h treatment with PDGF, as observed above. However, the addition of dex induced the frameshift MMTV-*myc* expression without any concomitant increase in *mrl* RNA levels (Fig. 7B). In a cell line that harbored the normal MMTV-*myc* gene (XhR1), dex induced a 3.5-fold increase in *mrl* mRNA levels over that elicited by PDGF, when equalized to actin levels (Fig. 7B). This result is similar to that obtained with 9E MMTV-*myc* fibroblasts, and the increase is apparent even though the induction of MMTV-*myc* in XhR1 was less after a 2-h treatment with dex than in either 9E or the Bst2 frameshift line. Thus, the protein-coding capacity of the exogenous MMTV-*myc* gene is essential for the ability of dex to regulate *mrl*. We conclude that the c-*myc* protein rapidly regulates steady-state *mrl* mRNA levels in PDGF-stimulated BALB/c cells.

Dex has no effect on *mrl* transcription in MMTV-*myc* cells. The rapid and specific regulation of *mrl* by c-*myc* protein in 9E cells prompted us to analyze *mrl* transcription. The nuclear run-on assay was used to examine the transcriptional state of *mrl*, the PDGF-inducible sequence JE (9), and the housekeeping genes GADPH and histone 3. *Hae*III-digested lambda bacteriophage DNA was used as a negative control for hybridization, since the 9E cell line contains plasmid-derived sequences in transcriptionally active regions (i.e., at the exogenous MMTV-*myc* locus).

The results from run-on experiments with nuclei from 9E cells stimulated 2 h with either PDGF or PDGF and dex are shown in Fig. 8. PDGF increased *mrl* transcription ~3-fold within 2 h when normalized to the signal from GADPH. This induction was corroborated in run-on assays with BALB/c A31 cell nuclei, where modest stimulations were also observed (data not shown). The PDGF-inducible gene JE was also transcriptionally stimulated, but to a greater degree. After a 2-h stimulation with both PDGF and dex, however, we observe no change in *mrl* transcription above that induced by PDGF alone. Similar results were obtained with nuclei from stimulated XhR1 cells or 9E cells incubated in 5% PPP with or without dex (data not shown). The absence of changes in *mrl* transcription contrasted with the fivefold increase in *mrl* cytoplasmic RNA levels induced by the same conditions. Thus, the regulation of *mrl* by c-*myc* in PDGF-stimulated cells appears to occur at some posttranscriptional level.

Dex has no effect on cytoplasmic *mrl* RNA stability. To test the possibility that c-*myc* might indirectly regulate *mrl* at the level of cytoplasmic RNA stability, we compared the *mrl* mRNA half-life in 9E MMTV-*myc* cells stimulated for 2 h with PDGF or PDGF plus dex by analysis of RNA decay in stimulated cells treated for various times with the transcription inhibitor actinomycin D (Fig. 9). Nearly identical RNA half-lives were found for *mrl* in PDGF-stimulated (50 min) and PDGF-dex-stimulated (56 min) 9E cells. Therefore, there is no significant difference in mRNA half-life elicited by dex in PDGF-stimulated 9E cells. We conclude that c-*myc* regulates the level of *mrl* mRNA at some posttranscriptional step other than stabilization of cytoplasmic RNA.

DISCUSSION

Identification of cellular genes deregulated by the c-*myc* oncogene. The differential cDNA hybridization screen used to identify genes deregulated by constitutive c-*myc* expression rested on two major assumptions. First, we assumed that c-*myc* could specifically modulate cellular gene expression that could be monitored at the level of cytoplasmic RNA. This was loosely based on the observation that the induction of c-*myc* by PDGF as G_0 cells become competent to enter the cell cycle parallels the de novo expression of sequences representing ~0.3% of the total number of genes transcribed in 3T3 cells (44). Furthermore, c-*myc* can at least partially relieve the requirement for some exogenous growth factors (10). Since the c-*myc* protein localizes to the nucleus (16, 36, 42), it is reasonable to suppose that it carries out its function by regulating genes transcriptionally or posttranscriptionally. Our second assumption was that, although both E1a and c-*myc* control growth, there would exist a

FIG. 7. Frameshift mutation in MMTV-*myc* eliminates the induction of *mrl* by dex. (A) Maps of the MMTV-*myc* constructs. At the top is the pXhR/*his* construct carrying an *Xho*I-*Eco*RI human genomic c-*myc* DNA fragment fused to the MMTV promoter. At the bottom is the pXhR/Bst construct carrying the frameshift mutation at the *Bst*EII site in the human c-*myc* second exon. The sequence of the mutation created and the predicted amino acid sequence of the frameshift region are shown. Restriction enzyme abbreviations: H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Xh, *Xho*I; Bs, *Bst*EII; E, *Eco*RI. (B) The BALB/c MMTV-*myc* lines XhR1 and Bst2, carrying the MMTV-*myc* constructs pXhR/*his* and pXhR/Bst, respectively, were made quiescent in DMEM containing 0.3% fetal calf serum. RNA was prepared from quiescent cells or those treated for 2 h with PDGF with or without dex and analyzed by Northern blotting as in Fig. 1B. A duplicate Northern blot of equivalent amounts of RNA was used to generate the panel showing exogeneous c-*myc* expression.

subset of *myc*-responsive genes that were not also modulated by E1a. There are several lines of circumstantial evidence to support this hypothesis. (i) Expression of several cellular genes associated with the early phases of the cell cycle, the so-called competence genes, are low in E1a-immortalized BRKs (21). (ii) E1a preferentially induces a subset of serum-inducible genes normally activated in the late G_1 or S phase of the cell cycle (30). (iii) E1a-immortalized BRKs are substantially growth factor independent in short-term assays and arrest poorly at confluence (21). In fact, it has been reported that RNA synthesis inhibitors do not prevent E1a-induced cellular DNA synthesis in semipermissive rodent cells infected with adenovirus (27). Thus, it appears that E1a-immortalized cells are independent of many signals required by normal cells in the G_0 phase to reenter the cell cycle (35). This is in contrast to *myc*-immoritalized fibroblasts, which continue to be substantially dependent on growth factors for DNA synthesis and cell division (21).

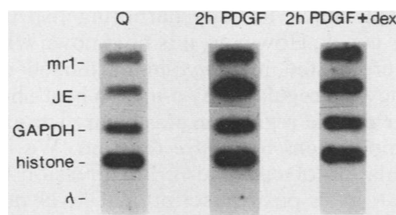

FIG. 8. dex has no effect on *mrl* transcription in 9E MMTV-*myc* fibroblasts. 9E cell nuclei from quiescent cells or those treated for 2 h with PDGF with or without dex were processed and analyzed as in Fig. 5. DNA targets for hybridization are 8 μg of linearized plasmid containing *mrl* cDNA, JE cDNA, avian GAPDH, mouse histone 3, and *Hae*III-digested lambda bacteriophage DNA (λ).

Use of the immortalized BRK cell system in identifying c-*myc*-regulated genes was valuable for three reasons. First, as mentioned above, the tight correlation between c-*myc* expression and growth was uncoupled in E1a BRK cells. Thus, the differential hybridization would screen for differences in gene expression correlated with c-*myc* levels but not with the far larger set of genes whose expression correlates with entrance to the S phase. Second, since the abundance of c-*myc* RNA in the *myc*BRK lines was quite high (30- to 50-fold above that in the E1aBRK lines), genes sensitive to higher c-*myc* levels would be more readily

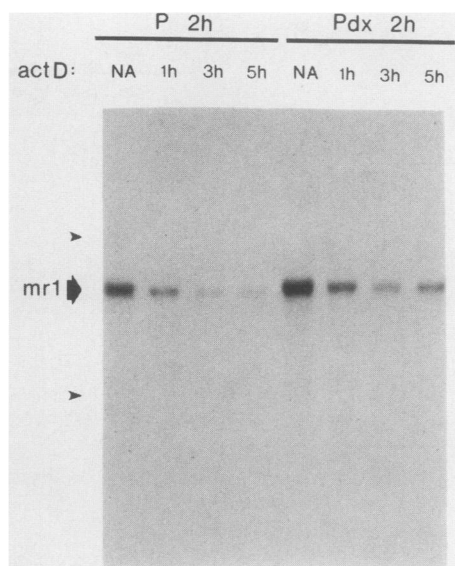

FIG. 9. dex has no effect on *mr1* cytoplasmic message stability in 9E MMTV-*myc* fibroblasts. Quiescent 9E cells were stimulated for 2 h with PDGF (P) with or without dex, as in Fig. 1B, and then the transcription inhibitor actinomycin D was added to a final concentration of 5 μg/ml. RNA was prepared at the indicated times after actinomycin D addition and examined by Northern analysis.

identified. Thus, the use of cDNA probes with widely divergent c-*myc* levels would increase the likelihood of detecting a low-abundance, dose-dependent responsive message. Finally, this system allows the use of multiple cDNA probes from the same cell type, allowing clones to be selected that consistently correlated with c-*myc* levels. This resulted in the selection of a set of clones that was 0.05% of the total number screened, a 150-fold enrichment over the number isolated by using only two probes (~1.5%).

c-*myc* immortalization induces changes in gene expression which functionally relate to the proliferative response. The results presented here define at least two molecular consequences of immortalization by c-*myc* which do not overlap with E1a, supporting our previous study indicating that E1a and c-*myc* have different effects on cellular growth pathways (21). First, we found that c-*myc* immortalization of primary rodent fibroblasts produced changes in cellular gene expression that functionally related to proliferative responses: *mr1* and *mr2* encoded cellular genes responding to immortalization by c-*myc*, but not by E1a, and these same genes were regulated in established cell lines by serum factors which controlled entrance to the cell cycle. Second, both *mr1* and *mr2* were constitutively expressed in both subconfluent and confluent *myc*BRK cultures but not in E1aBRK or the established REF52 fibroblast cell line. These observations suggest that *mr1* and *mr2* may be functionally involved in c-*myc* immortalization of BRK cells, since immortalization is correlated with somewhat decreased growth factor requirements (21). Studies of the function and regulation of *mr1* and *mr2* in *onc*-immortalized cells may contribute to an understanding of the pathways used in signal transduction which are relevant to normal and transformed cellular proliferative responses.

It should be noted that the levels of *mr1* and *mr2* did not parallel c-*myc* expression in subconfluent BRK cultures; i.e., expression was high in both *myc*- and E1a-immortalized cells despite very different levels of c-*myc*. Two possible explanations can account for this observation. If c-*myc* expression is a necessary condition for the expression of these genes in subconfluent cells, the low levels of c-*myc* in the proliferating cells may be adequate for their proper regulation. If c-*myc* expression is not a necessary condition for expression of these genes in subconfluent cells, then *mr1* and *mr2* could be completely independent of c-*myc* in growing cells. These propositions are difficult to distinguish since there is no method for generating a c-*myc* null cell. However, we argue that in confluent cultures c-*myc* expression is a sufficient condition of expression, since *mr1* and *mr2* are deregulated under these conditions in c-*myc*-immortalized BRKs.

Molecular evidence that PDGF and c-*myc* jointly control gene regulation events during the G_0-G_1 transition in BALB/c fibroblasts. Our results provide molecular evidence that c-*myc* can cooperate with other PDGF-induced factors to regulate genes as quiescent 3T3 cells become competent (37) to enter the cell cycle. These results complement the biological evidence from several studies, indicating that c-*myc* plays an accessory but perhaps not a central role in PDGF-stimulated DNA synthesis (1, 5, 11, 41). c-*myc* expression can induce entry into the S phase in a subpopulation of BALB/c cells grown in medium lacking PDGF, but it cannot induce cell proliferation (5). Other workers have reported that c-*myc* expression increases the response of BALB/c (1) and C310$T_{1/2}$ mouse fibroblasts (41) to PDGF, as measured by assays for replicative DNA synthesis and cell growth. However, c-*myc* cannot completely account for the mitogenic effects of PDGF in fibroblasts or bovine smooth muscle cells (11). Thus, the molecular data for *mr1* induction by c-*myc* presented above parallels the functional role determined for c-*myc* in these biological studies: we found that *mr1* was modestly induced by c-*myc* in quiescent, translationally competent fibroblasts, but this induction was small compared with that in the presence of PDGF.

There are 12 to 15 PDGF-inducible cellular mRNAs currently known (9, 25, 26). *mr1* appears to be a novel member of this class, since its mRNA size, kinetics of serum stimulation, and kinetics of transcriptional induction by PDGF do not coincide with those of any of the known genes (unpublished observations). In contrast to this group, *mr1* transcription is only modestly induced by PDGF (e.g., compare JE and *mr1* in Fig. 8) (27). Still, this modest stimulation of *mr1* transcription helps explain why c-*myc* induces the gene so weakly by itself, since a posttranscriptional regulation event controlled by c-*myc* would naturally be somewhat impotent in the face of transcriptional quiescence. Evidence for an intersection between regulatory targets for c-*myc* and serum-derived growth factors has also been recently reported (39). These workers found that two RNAs normally induced briefly during the G_0-G_1 cell cycle transition could be induced by heat shock in cells harboring hsp70 promoter-driven c-*myc* genes. However, it is not known whether these genes are deregulated in c-*myc*-immortalized cells nor at what level they are regulated by c-*myc* in heat-shocked cells.

Posttranscriptional regulation of cellular gene expression by c-*myc* and implications for c-*myc* function. We have shown that the regulation of *mr1* and *mr2* expression by c-*myc* is manifested at some posttranscriptional level not including mRNA stability. It remains to be determined whether expression of these genes is controlled directly or indirectly by c-*myc*. However, the rapidity and specificity with which c-*myc* activates *mr1* expression suggests that there may be a direct interaction between these genes. It is interesting to compare the results presented here with a recent immuno-

fluorescence study in which c-*myc* protein was found to be associated with an RNase-sensitive nuclear region in cells expressing high c-*myc* levels (42). These authors suggested that c-*myc* may be involved with cellular RNA metabolism, since the nuclear staining pattern depended on the integrity of nuclear RNA and not DNA and was similar to that found with antisera to small nuclear ribonucleoproteins. An interaction between the c-*myc* polypeptide and nuclear RNA is intriguing in relation to our finding that the protein posttranscriptionally regulates a cellular mRNA. If c-*myc* regulates *mrl* directly, then the function of the c-*myc* protein may be to modulate gene expression at a posttranscriptional step in the nucleus (e.g., RNA processing, turnover, nucleocytoplasmic transport). One prediction of this model is a biochemical association between the c-*myc* protein and target RNAs like *mrl* in the nucleus. Experiments to test this prediction will determine whether such a direct interaction exists.

The model for c-*myc* function presented here contrasts with other models in which c-*myc* is proposed to function in DNA replication (7) or as a transcriptional regulator (18). In the latter study, c-*myc* expression is reported to stimulate transcription from the hsp70 promoter and inhibit it from the metallothionein I promoter in transient BALB/c cotransfection assays. However, in both c-*myc*-immortalized fibroblasts and BALB/c MMTV-*myc* cells we have observed that increased c-*myc* levels correlate with neither increased hsp70 mRNA nor transcription levels (unpublished observations). This discrepancy may be due to differences in the assays for c-*myc* function, the level of regulation (i.e., direct or indirect), or other unknown factors.

The major activity of the c-*myc* gene that has been investigated to date is that of its influence on proliferative potential. However, several recent reports indicate that c-*myc* may be activated during differentiation in vitro, concomitantly with a decrease in proliferative protential in these systems (13, 23, 24, 34, 40). A model proposing that c-*myc* is a posttranscriptional regulator offers a potential explanation for the diverse biological effects of c-*myc* expression in different cellular backgrounds. Since one might imagine that transcriptional controls are dominant, the set of genes actually controllable by c-*myc* at any stage would only be a subset of those potentially controllable. Expression of the c-*myc* gene could have a variety of physiological consequences in different tissues simply because different subsets of target genes are transcriptionally active. In this way, the same oncoprotein activity could manifest a variety of cellular responses (e.g., proliferation or differentiation) as a consequence of the transcriptional background that is operational. This scenario also suggests that c-*myc* may be capable of performing as a dominant oncogene only in those cellular states where the subset of its target genes that affect transformation parameters are transcriptionally active.

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