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Biochemistry

c-Myc transactivation of *LDH-A*: Implications for tumor metabolism and growth

(oncogene/lactate dehydrogenase/hypoxia/tumorigenicity)

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ABSTRACT **Cancer cells are able to overproduce lactic acid aerobically, whereas normal cells undergo anaerobic glycolysis only when deprived of oxygen. Tumor aerobic glycolysis was recognized about seven decades ago; however, its molecular basis has remained elusive. The lactate dehydrogenase-A gene (*LDH-A*), whose product participates in normal anaerobic glycolysis and is frequently increased in human cancers, was identified as a c-Myc-responsive gene. Stably transfected Rat1a fibroblasts that overexpress *LDH-A* alone or those transformed by c-Myc overproduce lactic acid. *LDH-A* overexpression is required for c-Myc-mediated transformation because lowering its level through antisense *LDH-A* expression reduces soft agar clonogenicity of c-Myc-transformed Rat1a fibroblasts, c-Myc-transformed human lymphoblastoid cells, and Burkitt lymphoma cells. Although antisense expression of LDH-A did not affect the growth of c-Myc-transformed fibroblasts adherent to culture dishes under normoxic conditions, the growth of these adherent cells in hypoxia was reduced. These observations suggest that an increased LDH-A level is required for the growth of a transformed spheroid cell mass, which has a hypoxic internal microenvironment. Our studies have linked c-Myc to the induction of *LDH-A*, whose expression increases lactate production and is necessary for c-Myc-mediated transformation.**

Alterations of the glycolytic pathway including elevation of lactate dehydrogenase-A (LDH-A) activity are thought to be hallmarks of cancer cells, which unlike normal cells are able to produce lactate aerobically, a phenomenon known as the Warburg effect (1–4). The *LDH-A* gene is an epidermal growth factor, cAMP and phorbol ester-inducible delayed early serum response gene whose role in neoplasia remains unestablished despite its widespread use as a prognostic tumor marker (5–8). LDH is a tetrameric enzyme with five isoforms composed of combinations of two subunits, LDH-A and LDH-B. The LDH-A subunit converts pyruvate to lactate under anaerobic conditions in normal cells. The other isoenzyme, LDH-B, kinetically favors the conversion of lactate to pyruvate and is found at high levels in aerobic tissues such as the heart. Hereditary LDH-A subunit deficiency causes early postimplantation embryonic lethality in homozygotic mice (9) and an exertional myopathy in LDH-A-deficient humans (10). In addition to its role in intermediary metabolism, the LDH-A isozyme may be functionally involved in the transcriptional modulation of gene expression and/or DNA replication (11–15).

c-*myc* is an early serum response gene whose deregulated expression is the molecular signature of Burkitt lymphomas and is frequently found in various human and animal tumors (16–19). The c-Myc protein participates in the regulation of cell proliferation, differentiation, and apoptosis (20, 21). c-Myc is a basic helix–loop–helix (HLH)/leucine zipper transcription factor that heterodimerizes with another protein termed Max through the HLH/leucine zipper domain to bind a DNA consensus core sequence, CACGTG or E-box (22–24). Although c-Myc provides the transregulatory function for the heterodimer to activate through E-boxes or suppress gene transcription through initiator elements, its targets have not been comprehensively characterized and are only beginning to emerge from various studies (25–35).

Using representational difference analysis (RDA), we found that *LDH-A* is a direct c-Myc-responsive gene that is involved in Myc-mediated cell transformation (36, 37). We observed that overexpression of LDH-A alone is sufficient to increase lactate production. Reduction of LDH-A levels abrogates soft agar clonogenicity of c-Myc-transformed cells, a result that, together with our other observations, suggests that overexpression of LDH-A contributes to altered metabolism, which confers neoplastic growth advantage.

EXPERIMENTAL PROCEDURES

Description of Plasmids. The rat *LDH-A* sense expression vector consists of an *Eco*RI/*Bgl*II 1.6-kb *LDH-A* cDNA fragment (7) subcloned into pSG5, a simian virus 40 promoter expression vector (Stratagene). Antisense pSG5 vector consists of the same *LDH-A* cDNA fragment ligated to pSG5 *Nde*I/*Bgl*II and pSG5 *Nde*I/*Eco*RI fragments. An Epstein–Barr virus-origin episomal antisense *LDH-A* expression vector for lymphoid cells was constructed from an *LDH-A* cDNA *Hin*dIII/*Not*I fragment, pHEBoCMVneo *Sst*I/*Hin*dIII and pHEBoCMVneo *Sst*I/*Not*I fragments (38). The rat *LDH-A* *Xba*I-restricted 642-bp promoter (−1173/+25 bp; GenBank/EMBL Data Bank accession no. U05674) was subcloned into a luciferase reporter, pGL2Luc (Promega), to generate pGLDH637Luc (8). The two *LDH-A* promoter Myc/Max E-boxes, CACGTG, were mutated to CCCGGG by PCR-assisted methods. The 5′ E-box was mutated by using a pair of primers: 5′-TTGGGGTGTCGCAGCACCCGGGGAG-CCACTCTTGCAGG and 5′-TCTAGACGCAGAGCAGC-ACG. The mutated, PCR-amplified fragment was then used in a PCR as the 3′ primer with another 5′ primer: 5′-CTGCT-ATGGCGGATAGACC. The mutated *Aat*II/*Nsi*I promoter fragment was shuttled by TA cloning (Invitrogen) and subcloned back into the reporter construct, pGLDH637Luc. The

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Abbreviations: RDA, representational difference analysis; ER, estrogen receptor; CHX, cycloheximide; 4-HOTM, 4-hydroxytamoxifen.

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3′ E-box was mutated by using a pair of primers: 5′-CTGCTATGGCGGATAGACC and 5′-TGCGGGAACCCCCGGGTAGGCTGGGCCG. The double-E-box mutant was made by combining the single-E-box mutants through one of two *Bss*HII restriction sites and a flanking *Eco*RI site.

RDA. Rat1a or Rat1a-Myc cell lines (2×10^6 cells per 150-mm plate) were grown on a layer of 0.7% agarose in DMEM for 48 h. Total RNA was isolated by guanidium thiocyanate lysis followed by cesium chloride centrifugation. Poly(A)$^+$ mRNAs were selected on an oligo(dT) column. cDNA was synthesized by using a kit (Promega). cDNAs were digested with *Dpn*II and subjected to RDA as described (36, 37). RDA-selected amplicons were subcloned directly into pCR 2.1 vector by TA cloning.

RNA Analyses and Nuclear Run-On Assays. Total RNA (15 μg) was used in RNase protection assay (RPA II kit; Ambion, Austin, TX). Rat vimentin mRNA levels were independent of Myc expression as determined by Northern blot analysis; therefore, vimentin was used as an internal control. Northern blot analyses were performed as described (39).

Nuclear run-on assays were performed essentially as described (40). Nuclei (2×10^7) were incubated with buffered [^{32}P]UTP (150 μCi; 1 Ci = 37 GBq), and labeled RNAs were isolated after DNase treatment, proteinase K incubation, and phenol-chloroform extraction. Labeled RNAs (3×10^7 cpm) from either Rat1a-Myc or Rat1a cells were hybridized at 42°C for 48 h with membrane slot-blotted denatured LDH-A and vimentin cDNA fragments and washed five times with 0.1× standard saline citrate (SSC)/0.1% SDS at 50°C every 20 min.

Luciferase Assay. Luciferase assays (Promega) were performed, and the data were normalized for total protein as measured by the method of Bradford (41).

Immunoblotting. Equivalent amounts of total cellular proteins were resolved by SDS/PAGE (10% gel) and subjected to immunoblot analysis (42). Monoclonal mouse anti-Myc antibody 9E10 (1:2000 dilution) (43) and polyclonal anti-human Bcl-2 antibody (1:1000 dilution; PharMingen) were used.

LDH Enzyme Assay and Glucose–Lactate Measurements. The cell lysates (15 mM KCl, 10 mM Tris·HCl, 1.5 mM MgCl2, and 6 mM mercaptoethanol at pH 7.4) were centrifuged at $16{,}000 \times g$ for 5 min, and the supernatants were collected for LDH enzyme assays (Sigma) (44). Glucose and lactate levels in culture media were measured with a YSI model 2300 Stat Glucose/Lactate Analyzer (Yellow Springs, OH).

Cell Culture and Transfection. Rat fibroblast cells were cultured in 5% CO_2 at 37°C by using DMEM supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL) and antibiotics. Human lymphoid cells were cultured in Iscove's modified Dulbecco's medium, and human lung carcinoma H209 cells were cultured in RPMI 1640 medium. Rat1a fibroblasts were transfected with pSG5–*LDH-A* sense or antisense and a puromycin-resistance marker plasmid (pBABE-puro) by using Lipofectin (GIBCO/BRL) (39). Pooled transfected Rat1a cells were selected with 0.75 μg/ml puromycin. Lymphoid cells were lipofected with pHEBoCMVneo vectors and selected in 800 μg/ml G418. A Rat1a cell line expressing the c-Myc–estrogen receptor (ER) fusion protein (a gift of J. M. Bishop, University of California, San Francisco) was passaged in DMEM with 10% FBS and cultured at 80% confluency in DMEM without phenol red (GIBCO/BRL) and 10% charcoal-treated FBS (HyClone) for 48 h before induction of Myc (45). To induce c-Myc activity, Myc–ER cells grown to confluency were exposed to 0.25 μM 4-hydroxytamoxifen (4-HOTM; Research Biochemicals, Natrick, MA) for the indicated times as described (29). To block protein synthesis, these cells were exposed to 10 μM cycloheximide (CHX) 30 min before addition of 4-HOTM.

For hypoxic treatment, cells were cultured in DMEM (6 ml per 100-mm dish) with 25 mM Hepes (pH 7.55) and 10% FBS for 48 h in hypoxic chambers (46). The chambers were sealed and gassed daily with 5% CO_2 and 95% N_2.

Growth rates of lymphoid cells and fibroblasts were determined by plating 5×10^4 and 1×10^4 cells, respectively, per 60-mm dish. At the indicated time points, cells from triplicate dishes were counted in a Coulter counter.

Soft Agar Clonogenic and Focus Formation Assays. The soft agar assay was performed as described (39). For lymphoid cells, soft agar assays were performed with 5×10^5 cells in 0.3% top agarose with Iscove's modified Dulbecco's medium as described (38). Rat1a cells were lipofected with pEJ-ras (2 μg per 100-mm dish) encoding activated H-ras and either the empty pSG5 expression plasmid (4 μg per 100-mm dish) or the LDH-A antisense expression plasmid pAS–LDH-A (4 μg per 100-mm dish). Transformed foci were determined by photography at 2 weeks after transfection. For the colony suppression assay, either the empty pSG5 expression plasmid (4 μg per 100-mm dish) or the LDH-A antisense expression plasmid pAS–LDH-A (4 μg per 100-mm dish) was lipofected with the pBABE-puro (0.2 μg per 100-mm dish). Puromycin-resistant colonies were counted in triplicate experiments 7 days after the beginning of drug selection.

Flow Cytometric Apoptosis Assay. DNA fragmentation characteristic of apoptosis was quantified by using two-dimensional flow cytometry (47). Cells were fixed in 1% formaldehyde followed by 70% methanol, washed, and incubated at 37°C with the deoxynucleotide analog biotin-16-dUTP plus terminal deoxynucleotidyltransferase (Boehringer Mannheim). Cells were then treated with a fluorescein isothiocyanate-conjugated avidin (Boehringer Mannheim), followed by propidium iodide staining, and analyzed by flow cytometry as described above.

RESULTS

***LDH-A* Is a Target of c-Myc.** To identify potential c-Myc target genes, we used Rat1a fibroblasts that only require ectopic c-Myc expression to display anchorage-independent growth, whereas nontransformed cells require adherence for cell proliferation (48, 49). We synthesized cDNAs from nonadherent Rat1a-Myc and Rat1a cells and used cDNA RDA to identify differentially expressed genes that are regulated by c-Myc and contribute to the anchorage-independent growth phenotype (36, 37). Among 20 differentially expressed genes (B.C.L., H.S., Q. Li, C.-S.W., L. Lee, A. Maity, and C.V.D., unpublished results), *LDH-A* proved to be one that is highly differentially expressed, displaying a 6.7(± 0.7)-fold elevated expression in Myc-transformed Rat1a cells (Fig. 1*A*). Nuclear run-on experiments (Fig. 1*B*) demonstrated an enhanced transcriptional rate of *LDH-A* in Rat1a-Myc cells as

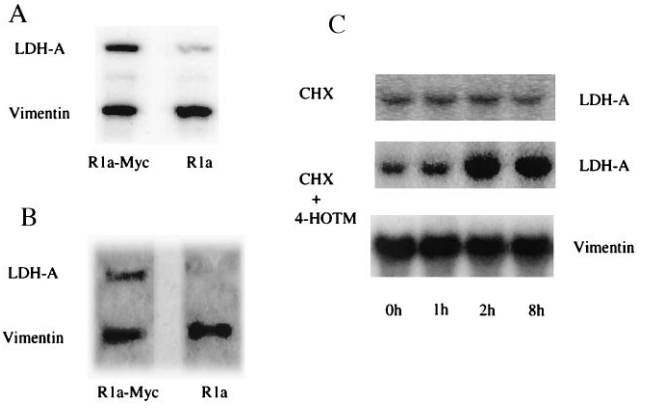

FIG. 1. *LDH-A* is a direct c-Myc target gene. (*A*) RNase protection assay showing elevated *LDH-A* expression (320-bp protected band) in nonadherent Rat1a-Myc (R1a-Myc) versus Rat1a (R1a) cells. (*B*) Nuclear run-on assays with isolated nuclei from nonadherent cells demonstrate an increased transcriptional rate of *LDH-A* in Rat1a-Myc as compared with Rat1a cells. (*C*) Northern blots showing LDH-A expression in a cell line expressing a Myc–ER fusion protein on exposure to CHX alone (*Top*) or exposure to both CHX and 4-HOTM (*Middle*) for the indicated times. The -fold change of LDH-A expression relative to vimentin mRNA control was as follows: 1.0, 0 h; 0.9, 1 h; 2.5, 2 h; 2.5, 8 h.

compared with Rat1a fibroblasts. To determine whether the *LDH-A* gene is transcriptionally activated by Myc, we used a previously described Rat1a cell line expressing a Myc–ER fusion protein that is activated by the addition of 4-HOTM to the growth medium (29, 45). Activation of Myc–ER by 4-HOTM causes induction of *LDH-A*, which is neither inhibited nor activated by the protein synthesis inhibitor CHX (Fig. 1*C*). These observations suggest that induction of *LDH-A* expression by Myc is direct and does not require new protein synthesis.

We observed a correlation between c-Myc expression and the levels of LDH-A mRNA and activity in lymphoid cells (Fig. 2). Nontransformed CB33 lymphoblastoid cells, those transformed by c-Myc (CB33-Myc), and three Burkitt lymphoma cell lines with c-*myc* chromosomal translocations were studied. The c-Myc-transformed lymphoblastoid and Burkitt lymphoma cell lines all have elevated c-Myc protein levels that are associated with elevations of LDH-A mRNA (1.7 kb) and enzymatic levels compared with the nontransformed lymphoblastoid cells. The lower band in Fig. 2*C* corresponds to LDH-B mRNA (1.4 kb) that cross-hybridized with randomly primed rat LDH-A radiolabeled probes (13). Although elevated c-Myc protein levels are correlated with elevated LDH-A mRNA and activity, the ST486 cell line has a high level of c-Myc protein but a relatively lower amount of LDH-A mRNA. Our observations indicate that c-Myc increases the expression of LDH-A, which may be modulated by other factors, and augments *LDH-A* mRNA in Rat1a fibroblasts at the transcriptional level.

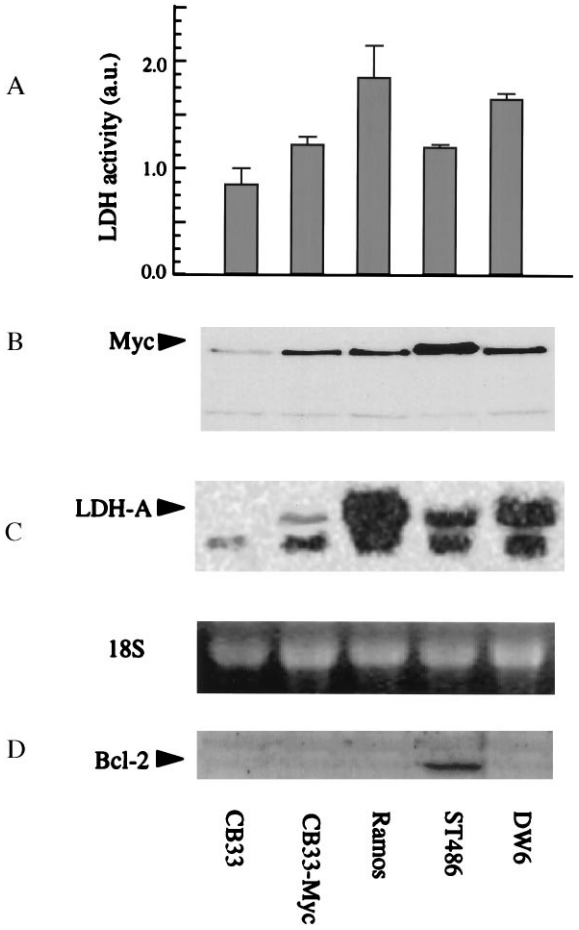

FIG. 2. Deregulated c-Myc expression elevates LDH-A mRNA and activity in lymphoid cells. (*A*) Relative LDH enzymatic activities (a.u., arbitrary units) for CB33 lymphoblastoid cells (first bar), c-Myc-transformed CB33 cells (second bar), Ramos (third bar), ST486 (fourth bar), and DW6 (fifth bar) Burkitt lymphoma cell lines are shown. (*B*) The corresponding c-Myc protein levels determined by immunoblot analysis for each cell line are shown. (*C*) Northern blot showing LDH-A mRNA (1.7 kb) levels in the indicated cell lines (listed at the bottom of the figure); 15 μg of total RNA was loaded per lane, and an ethidium bromide stained gel with the 18S ribosomal RNA band is shown as control for sample loading. The -fold changes of LDH-A mRNA (corrected with 18S RNA) were as follows: CB33, 1.0; CB33-Myc, 2.0; Ramos, 18.2; ST486, 5.5; and DW6, 10.6. (*D*) Bcl-2 protein levels by immunoblot analysis are shown for each cell line. Note that the ST486 cell line (lane 4) displayed large amounts of Bcl-2 protein as compared with CB33 lymphoblastoid (lanes 1 and 2) and the other Burkitt cells (lanes 3 and 5).

The rat *LDH-A* promoter contains two consensus Myc/Max binding sites or E-boxes, CACGTG (located at −78 through −83 and −175 through −180 from the transcriptional start site), that are conserved in mouse and man, suggesting that c-Myc may regulate the transcription of *LDH-A* through these sites (8, 50). Transient transfection experiments with a c-Myc expression vector demonstrated an E-box-dependent transactivation of the *LDH-A* promoter–luciferase reporter gene (Fig. 3). Mutation of either or both E-boxes (Fig. 3) abrogated Myc-dependent transactivation. The expression vector producing a c-Myc mutant lacking the HLH domain was unable to activate the *LDH-A* promoter (Fig. 3), suggesting that dimerization with Max is required for transactivation. An expression vector for the nononcogenic basic HLH/leucine zipper transcription factor USF (51), which also binds CACGTG, was also able to stimulate the *LDH-A* promoter, although only half as efficiently as the c-Myc expression vector (Fig. 3). *In vitro* electrophoretic mobility-shift DNA-protein binding assays demonstrated the ability of recombinant Myc/Max proteins to bind the *LDH-A* promoter E-boxes (data not shown). These

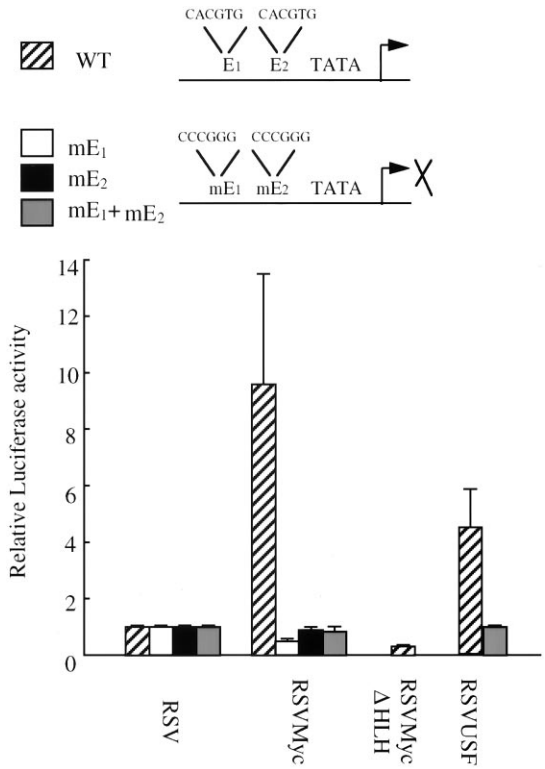

FIG. 3. c-Myc is able to transactivate the rat *LDH-A* promoter in an E-box-dependent manner in NIH 3T3 cells. The top diagram depicts the wild-type *LDH-A* promoter with two E-boxes (E_1 and E_2) and the corresponding mutations of the E-boxes, mE_1 and mE_2. The graph shows wild-type and mutant *LDH-A* promoter-reporter responses to empty vector (RSV) or a c-Myc (RSVMyc) expression vector. The responses of the wild-type LDH-A promoter to a Myc mutant lacking the HLH region (RSVMycΔHLH) and the transcription factor USF (RSVUSF) are also shown; there was no activation of the double-E-box mutant reporter. Empty vector, Myc, or USF expression plasmids were lipofected at 50 ng per 60-mm dish. Data are averages of four experiments with standard errors shown.

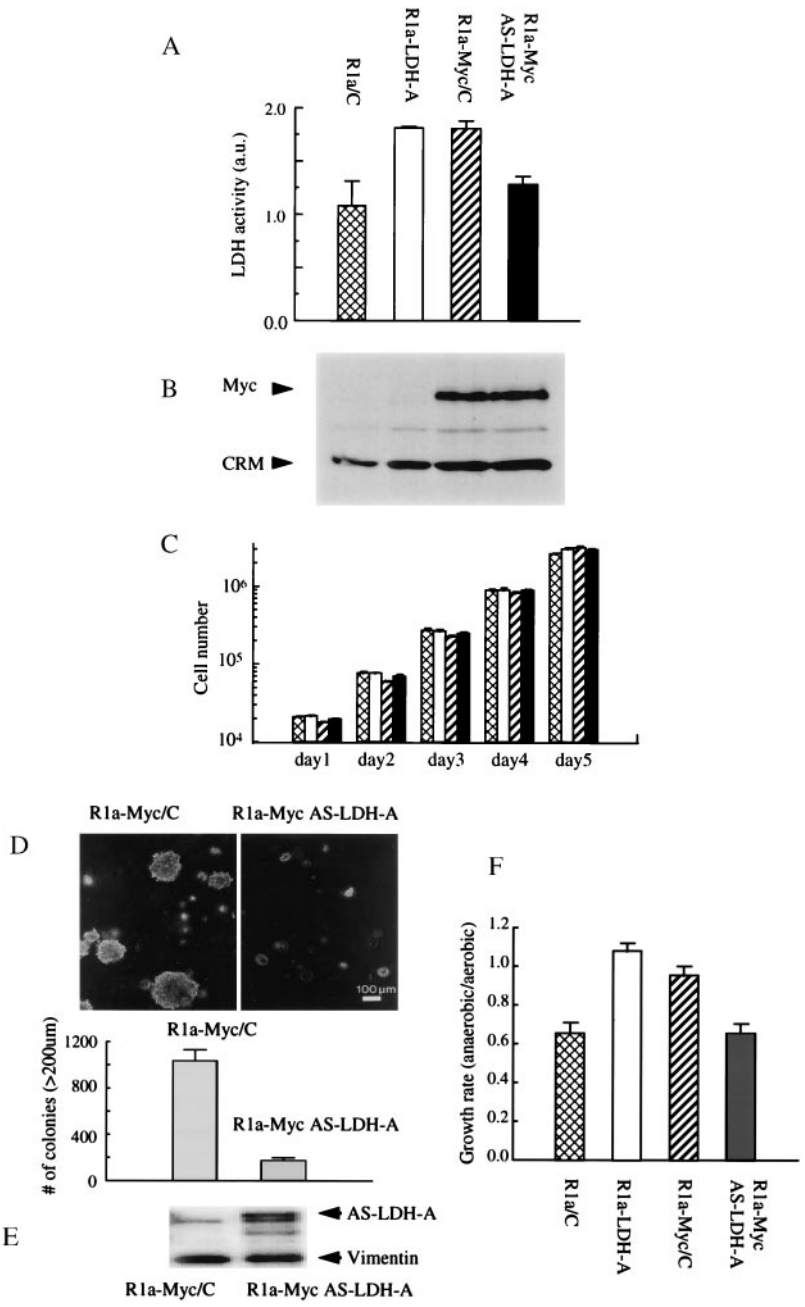

FIG. 4. Reduction of LDH-A expression in c-Myc-transformed cells inhibits anchorage-independent growth. (*A*) LDH-A enzymatic activities (mean of four experiments with standard error shown) of pooled Rat1a/C (R1a/C) puromycin-resistant control cells, pooled Rat1a-LDH-A (R1a-LDH-A) overexpressing *LDH-A*, pooled Rat1a-Myc/C (R1a-Myc/C) puromycin-resistant control cells, or pooled Rat1a-MycAS-LDH-A (R1a-MycAS-LDH-A) with antisense *LDH-A* expression are shown. (*B*) Immunoblot showing human c-Myc protein levels in pooled cell lines corresponding to those indicated in *A*. Expression of antisense *LDH-A* did not affect ectopic human c-Myc protein levels (compare third and fourth lanes). CRM, cross-reacting material. (*C*) The graph shows adherent growth rates of the four pooled cell lines indicated in *A*. The ordinate shows cell number per 60-mm dish (average of triplicate experiments with standard errors shown) at the indicated times. The shading of the bars corresponds to that shown in *A*. (*D*) Anchorage-independent growth assay of Rat1a-Myc/C cells and Rat1a-MycAS-LDH-A (photomicrograph) demonstrates a reduction in colony formation (graph) associated with decreased LDH-A levels. Data are averages of two experiments with standard error shown. (*E*) RNase protection assay demonstrating the expression of *LDH-A* antisense transcript (AS-LDH-A; 320 bp protected fragment using the *LDH-A* sense probe); vimentin was used as an internal control. (*F*) Comparative anaerobic-to-aerobic growth rates of cell lines described in *A*. Cells were grown adherently on 100-mm plastic dishes in a hypoxic chamber or in regular oxygenated conditions for 48 h, and cell numbers were determined from triplicate experiments.

observations and results with the Myc–ER system suggest that *LDH-A* is a direct Myc-responsive target gene.

Increased Expression of LDH-A Enhances Lactate Production. To determine whether LDH-A overexpression is sufficient to increase lactate production aerobically or transformation, Rat1a cells were stably transfected to constitutively express rat *LDH-A*. The Rat1a-LDH-A cells were unable to proliferate in soft agar as compared with Rat1a-Myc cells, indicating that increased *LDH-A* expression is not sufficient to fully induce transformation. We observed, however, that both c-Myc-transformed and *LDH-A* ectopically expressing Rat1a cells produce more lactate than the control Rat1a cells. The lactate production to glucose consumption molar ratios (average of four experiments) after 24 h of incubation with fresh medium were as follows: Rat1a, 1.23 ± 0.11; Rat1a-Myc, 1.46 ± 0.02; and Rat1a-LDH-A, 1.61 ± 0.05. These observations suggest that lactate overproduction induced in fibroblasts by c-Myc is largely due to the deregulated expression of *LDH-A*.

Necessity of Elevated LDH-A for c-Myc-Induced Soft Agar Growth of Fibroblasts and Lymphoid Cells. We determined whether elevated LDH-A expression is necessary for c-Myc-mediated anchorage-independent growth by constructing Rat1a-Myc cells and c-Myc-transformed lymphoblastoid cells expressing antisense *LDH-A* (Fig. 4*E*) (38). Reduction of LDH activity in Rat1a-Myc cells (Fig. 4*A*) did not alter ectopically expressed Myc protein levels (Fig. 4*B*) but dramatically decreased soft agar clonogenicity of Rat1a-Myc cells (Fig. 4*D*). The growth rates of Rat1a-Myc/C and those expressing antisense *LDH-A* (Rat1a-MycAS-LDH-A) were, however, virtually the same when they were grown adherent to plastic dishes (Fig. 4*C*). These results indicate that LDH-A is necessary for c-Myc-mediated, anchorage-independent growth of Rat1a cells, although *LDH-A* overexpression alone is insufficient to fully induce growth in soft agar.

We hypothesized that the anaerobic conditions within an expanding soft agar colony of cells competing for nutrients and oxygen may select against cells with low LDH-A levels and are thus inefficient in anaerobic glycolysis. To test this hypothesis, we subjected Rat1a/C, Rat1a-LDH-A, Rat1a-Myc/C, and Rat1a-MycAS-LDH-A cells cultured adherent to plastic dishes to hypoxia (Fig. 4*F*). Both Rat1a-Myc/C and Rat1a-LDH-A

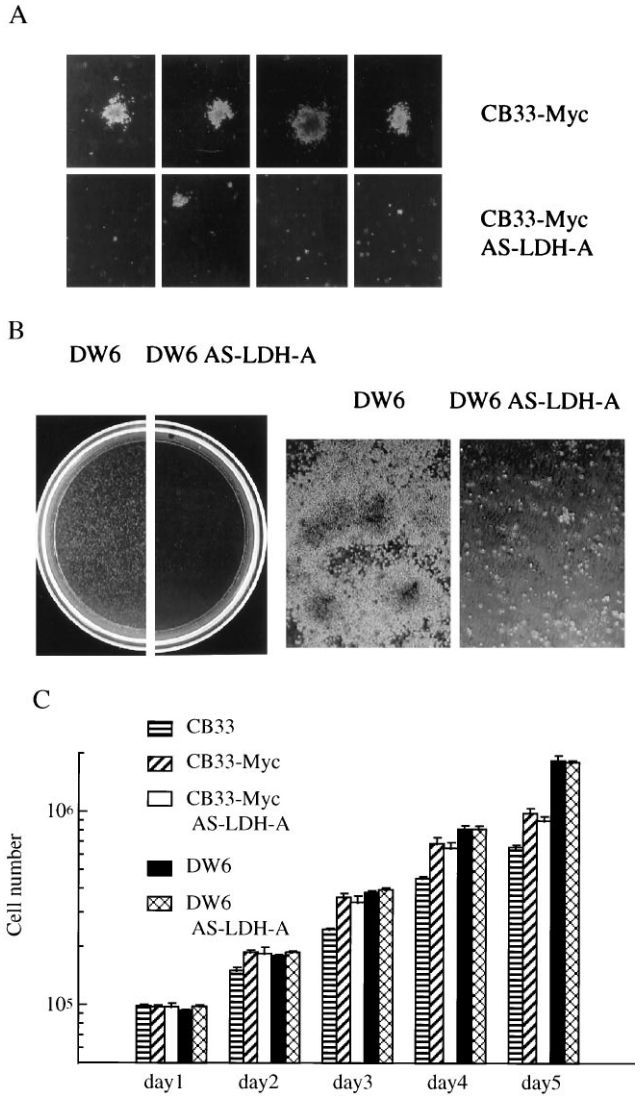

FIG. 5. (A) Antisense *LDH-A* expression reduced clonogenicity of c-Myc-transformed lymphoblastoid cells, CB33-Myc. Control, empty vector-transfected CB33-Myc cells were subjected to soft agar clonogenic assays. Representative colonies formed (cloning efficiency of 2×10^{-4}) are shown in the upper panels. With the antisense, *LDH-A*-expressing pooled clones, CB33-MycAS-LDH-A, there was a 4-fold reduction in cloning efficiency and a reduction in colony sizes. Representative microscopic fields are shown in the lower panels. (*B*) Antisense *LDH-A* expression reduced clonogenicity of the DW6 Burkitt lymphoma cell line. Pooled, stably transfected DW6 cells were cultured in soft agar. The left two panels represent a composite photograph of Petri dish halves containing DW6 cells transfected with empty vector (left half) or antisense *LDH-A* vector (right half). The right two panels are of higher magnification and show soft agar colonies of the same cells shown in the left two panels. (*C*) Growth rates of the indicated cell lines grown in suspension were determined. The ordinate shows cell numbers per 60-mm plate at the indicated times from triplicate experiments.

cells continued to grow for 48 h in hypoxic conditions as compared with the control Rat1a/C cells. Intriguingly, the Rat1a-MycAS-LDH-A cells had significantly reduced growth rates when deprived of oxygen. These observations support our hypothesis and provide a plausible biological basis for differences in the growth of these cells in soft agar.

In addition to the effects seen in fibroblasts, the mock-transfected, c-Myc-transformed lymphoblastoid cells form colonies in soft agar as previously reported (38), whereas cells transfected with antisense *LDH-A* showed a 4-fold reduction in soft agar cloning efficiency (Fig. 5*A*). The DW6 Burkitt lymphoma cell line displayed a very high soft agar cloning efficiency that was reduced more than 100-fold with antisense *LDH-A* expression (Fig. 5*B*). Both the DW6 and c-Myc-transformed lymphoblastoid CB33 cells that express antisense *LDH-A* display normal growth characteristics in standard suspension culture conditions (Fig. 5*C*). The Ramos Burkitt cell line displayed a much lower cloning efficiency, but its clonogenicity was 40-fold decreased with antisense *LDH-A* expression (data not shown). These results indicate that elevated LDH-A levels associated with overexpression of c-Myc are necessary for neoplastic transformation as measured by soft agar clonogenic assays.

We studied the effect of antisense LDH-A expression on activated Ras-mediated transformation of Rat1a cells to determine whether reduction of LDH-A affects other oncogene-induced transformation. Expression of activated Ras causes focus formation of Rat1a cells (778 ± 36 foci per 100-mm dish; $n = 3$) that was reduced 3.6-fold by coexpression of antisense LDH-A (214 ± 22 foci per 100-mm dish; $n = 3$). We further determined the potential toxicity of antisense LDH-A expression in a colony suppression assay to determine the contribution of toxicity to the suppression of Ras-mediated transformation. In this assay, puromycin-resistant colonies were counted after cotransfection of the puromycin-resistance marker gene with the empty expression vector or antisense LDH-A expression vector. The antisense LDH-A expression vector reduced puromycin-resistant colony formation by 40% as compared with control empty expression vector. These observations suggest that antisense LDH-A expression only slightly reduces (1.5-fold) the transforming potential of Ras. These effects, however, are quite different from the phenotypes observed with antisense LDH-A expression in stably transfected Myc-transformed cells. In the case of the Myc-transformed cells, the difference in phenotypes is profoundly different when cells are grown in soft agar.

Because deregulated c-Myc expression induces apoptosis in serum-deprived fibroblasts and LDH-A is downstream of c-Myc, we sought to determine whether elevated LDH-A causes c-Myc-induced apoptosis using the terminal deoxynucleotidyltransferase-mediated UTP end labeling assay (47). After 20 h of serum deprivation as previously described (39), Rat1a cells (3 ± 2% cell death) or Rat1a overexpressing LDH-A (6 ± 2% cell death) did not undergo apoptosis as did c-Myc-overexpressing cells (19 ± 3% cell death). Thus, elevated LDH-A does not appear to cause c-Myc-induced apoptosis of serum-deprived cells.

DISCUSSION

c-Myc Regulates the Expression of *LDH-A*. Our results indicate that c-Myc is able to transactivate the *LDH-A* promoter and directly increase *LDH-A* expression. The time course of serum induction of c-*myc* expression at 2 h followed by *LDH-A* expression at 4 h in fibroblasts is consistent with *LDH-A* being a target of c-Myc (7). Using cells expressing a Myc–ER fusion, we observed 4-HOTM induction of *LDH-A* expression that does not require new protein synthesis, suggesting that the induction of *LDH-A* by Myc is direct. We also observed a correlation between c-Myc overexpression and elevated LDH levels in c-Myc-transformed fibroblasts, and lymphoblastoid and Burkitt lymphoma cell lines. In addition, inducible c-Myc expression was previously reported to elevate *LDH-A* expression in two different fibroblast cell lines (52). These observations strongly support the hypothesis that *LDH-A* is a direct target of c-Myc. Because *LDH-A* expression is altered in response to various stimuli (5–8), it may also be regulated by other transcription factors such as USF (46, 51, 53). Our results demonstrate that *LDH-A* expression is elevated in c-Myc-transformed cells and suggest that *LDH-A* is a direct target of c-Myc.

Necessity of LDH-A Induction for Myc-Mediated Transformation. Reduced expression of LDH-A in our studies is able to block Myc-mediated soft agar colony formation but not anchorage-dependent growth, suggesting that elevated LDH-A expression in human cancers may be necessary for their neoplastic phenotype. We hypothesized that within an expanding soft agar

colony, the hypoxic condition resembling the microenvironment of cultured cellular spheroids (54) or tumor cells distal from blood vessels (55) may select against cells with low LDH-A levels and are inefficient in anaerobic glycolysis. This hypothesis is supported by our observations that LDH-A expression correlated with the ability of Rat1a-LDH-A and Rat1a-Myc, but not Rat1a or Rat1a-MycAS-LDH-A, to proliferate when adherent cells were cultured in hypoxic conditions. Our observation on the effect of hypoxia on stably transfected Rat1a cells constitutively expressing c-Myc contrasts with the previously reported hypoxia-induced apoptosis of cells expressing a Myc–ER fusion protein exposed to tamoxifen (56). In fact, we have also observed hypoxia-induced cell death with Myc–ER Rat1a cells upon exposure to 4-HOTM. This effect is enhanced when the growth medium is not buffered in hypoxic chambers to physiological pH. The key differences between the constitutive and inducible Myc-expressing Rat1a systems are as yet unresolved. Our hypothesis, however, is supported by the finding that the early embryonic lethality of homozygous LDH-A-deficient mice is probably due to the postimplantation anaerobic conditions (57) that exist before formation of the chorioallantoic placenta (9).

Seven decades ago, Warburg (1) found that there was a trend toward an increased rate of glycolysis in a variety of human and animal tumor cells, resulting in the excessive production of lactic acid from glucose. This phenomenon, known as the Warburg effect, was a subject of intense investigation, yet its molecular basis has remained unclear (4). Our results indicate that c-Myc is able to activate the expression of *LDH-A* and increase lactate production. Moreover, we observe that ectopic *LDH-A* expression is sufficient to increase lactate production in fibroblasts without conferring the fully transformed phenotype of anchorage-independent growth. Our findings may provide a molecular basis for altered tumor glycolysis when the Warburg effect and previous links between elevated LDH-A levels and human cancers are taken into consideration (2, 58, 59). In particular, an elevated LDH level is an independent predictor of poor clinical outcome in Burkitt lymphoma, a disease characterized by activation of the c-*myc* gene by chromosomal translocations (17, 59, 60). An elevation of lactate production in a transgenic mouse model that overexpresses c-Myc in the liver further supports the ability of c-Myc to induce the Warburg effect (61).

In summary, we surmise that the oncogenic transcription factor c-Myc acts pleiotropically to transform cells by up-regulating components of the cell cycle machinery such as CDC25A (27), stimulating the production of biosynthetic enzymes such as ornithine decarboxylase (26) to prepare cells for S-phase entry, and activating the expression of metabolic enzymes such as LDH-A to ensure an adequate supply of energy or signals for cell proliferation. This view readily explains why the retroviral v-*myc* oncogene (62) is sufficient to acutely cause avian tumors and why alterations of c-*myc* gene expression are frequently observed in human cancers.

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