A Novel c-Myc-responsive Gene, JPO1, Participates in Neoplastic Transformation*

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We have identified a novel c-Myc-responsive gene, named JPO1, by representation difference analysis. JPO1 responds to two inducible c-Myc systems and behaves as a direct c-Myc target gene. JPO1 mRNA expression is readily detectable in the thymus, small intestine, and colon, whereas expression is relatively low in spleen, bone marrow, and peripheral leukocytes. We cloned a full-length JPO1 cDNA that encodes a 47-kDa nuclear protein. To determine the role of JPO1 in Myc-mediated cellular phenotypes, stable Rat1a fibroblasts overexpressing JPO1 were tested and compared with transformed Rat1a-Myc cells. Although JPO1 has a diminished transforming activity as compared with c-Myc, JPO1 complements a transformation-defective Myc Box II mutant in the Rat1a transformation assay. This complementation provides evidence for a genetic link between c-Myc and JPO1. Similar to c-Myc, JPO1 overexpression enhances the clonogenicity of CB33 human lymphoblastoid cells in methylcellulose assays. These observations suggest that JPO1 participates in c-Myc-mediated transformation, supporting an emerging concept that c-Myc target genes constitute nodal points in a network of pathways that lead from c-Myc to various Myc-related phenotypes and ultimately to tumorigenesis.

c-myc was first identified as the cellular homologue of the v-myc oncogene in the avian myelocytomatosis retrovirus, MC29 (1). Since its discovery, c-myc has been implicated in numerous human and animal tumors (2, 3). In cases of Burkitt’s lymphoma, the c-myc gene on chromosome 8 is translocated to chromosome 2, 14, or 22 placing c-myc under the regulation of the immunoglobulin regulatory elements and leading to sustained elevated levels of c-Myc expression (4, 5). Elevated c-Myc expression also arises from genomic amplification of c-myc in breast cancer, lung, cervix, and colon carcinomas (6–8). More recently, mutations in the adenomatous polyposis coli (APC) pathway that lead to increased Myc levels have been identified in colon cancers (9–11).

c-Myc belongs to the family of basic helix-loop-helix leucine zipper transcription factors. c-Myc heterodimerizes with a partner protein, Max, through the C-terminal helix-loop-helix Zip domain (12, 13), and together Myc and Max bind DNA specifically (14, 15) to activate transcription through the core sequence 5’-CA(C/T)GTG-3’, also known as an E box (16, 17). The N-terminal transactivation domain of c-Myc contains two highly conserved regions among the Myc family of proteins; these regions are termed Myc Box I (c-Myc residues 45–63) and Myc Box II (c-Myc residues 128–143) (16). Despite the wealth of knowledge that has accumulated about c-Myc, the connection between elevated c-Myc levels and oncogenesis remains incompletely understood (18, 19). Presumably, the role of Myc as a transcription factor involves regulation of a set of genes that participate in the oncogenic process (20–22). A number of c-Myc target genes have been identified, including CAD (23), ODC (24, 25), LDH-A (26, 27), cyclin E (28), MrDb (29), telomerase/hTERT (30–35), rcl (36), IRP2 (37), and cdc25A (38). The use of cDNA microarrays has enlarged the list of potential c-Myc-responsive genes (39–42). Despite the long list of c-Myc-responsive genes, the biological effects of these genes in c-Myc related phenotypes are largely unknown.

Initial work in our laboratory relied on representational difference analysis (RDA) to identify novel putative c-Myc target genes that could be involved in cellular transformation. The screen made use of Rat1a fibroblasts that can be transformed by c-Myc alone (43). Unlike parental cells, Rat1a-Myc cells have an increased growth rate, are capable of anchorage-independent growth, form colonies in a soft agar assay (44), are tumorigenic, and undergo apoptosis in response to growth factor or glucose withdrawal (45, 46). Our original RDA screen identified 20 differentially expressed genes (36). We have named the most highly differentially expressed of these clones JPO1. Here we describe the cloning of human JPO1 and its characterization in the context of c-Myc-associated phenotypes. The role of JPO1 in transformation and anchorage-independent growth is underscored by its complementation with a transformation-defective c-Myc Box II mutant in Rat1a transformation assays.

EXPERIMENTAL PROCEDURES

Library Screening—An oligo(dT)- and random hexamer-primed liver and spleen cDNA library (CLONTECH, Palo Alto, CA) was screened with human EST 59390 that contained 912 bp of JPO1 cDNA. Of 4 ×
10^5 colonies screened, three independent overlapping positive clones were identified and purified according to the manufacturer’s directions, with the exception that it was necessary to reduce the temperature to washes of 50°C.

**RNase Protection Assay**—The partial rat JPO1 and rat vimentin sequencing cDNA cloned by RDA (36) were used as templates for RNA riboprobes. Probes were transcribed from linearized DNA using the T7 promoters in the pCR1 and pBluescript plasmids. Hybridization and RNase digestion were performed in accordance with an RPA kit from Ambion (Austin, TX). Digested samples were subjected to electrophoresis on an 8% urea, 6% polyacrylamide denaturing gel with Century Systems (CA). Hybridization was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Northern Blot Assay**—15 μg of total RNA isolated by Trizol (Life Technologies, Inc.) was subjected to electrophoresis on a 6% formaldehyde, 0.9% agarose gel and transferred to Nytran. Blots were hybridized with ≥10^6 counts/ml cDNA probes labeled with the PrimeIt II Random Primer Labeling Kit (Stratagene, La Jolla, CA). Rat JPO1 and rat vimentin probes were labeled from the partial clones identified by RDA. Human JPO1 probe was labeled from the partial cDNA clone contained in EST H59390. Rat c-myc was labeled from partial cDNAs. Hybridization was quantified by PhosphorImager (Molecular Dynamics).

**Western Blot Analysis**—Protein lysates were made by lysing cells in 10% SDS (10 cells/ml) and boiling lysates at 95°C for 5 min. Protein content was assayed by a BCA kit (Pierce), and equal amounts of protein were resuspended in 2x Laemmli buffer. Equal relative amounts of protein, ~10 μg, were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Protein blots were blocked with 5% milk, TBST and probed with antibodies diluted in the same. Anti-Myc 9E10 antibodies were diluted 1:7,000. Anti-Myc antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse antibodies diluted 1:10,000 in 5% milk/TBST. Protein blots were treated with enhanced chemiluminescent reagents and exposed to film. Equal loading was confirmed with cross-reacting bands and by Coomassie Blue staining of the gels.

**Tissue Culture**—Rat1a cells were grown in low glucose DMEM. TGR (myc +/+ ) and HO15 (myc −/− ) cells (47) were grown in high glucose DMEM. Rat1a-Myc cells (48, 49) were grown in 10% FCS DMEM supplemented with 10% charcoal-stripped fetal calf serum. CB33 cells (gift of R. Dalla-Favera, Columbia University, New York), an Epstein-Barr virus immortalized B lymphoblast line, CB33-derived cells, and Burkitt’s lymphoma cell lines (Ramos, Thomas, and Wynn) were grown in IMDM. COS7 cells and PG13 cells, a murine 3T3-based cells, and Burkitt Epstein-Barr virus immortalized B lymphoblast line, CB33 cells (gift of R. Dalla-Favera, Columbia University, New York), an Epstein-Barr virus immortalized B lymphoblast line, CB33-derived cells, and Burkitt’s lymphoma cell lines (Ramos, Thomas, and Wynn) were grown in IMDM. COS7 cells were grown in high glucose DMEM. All media contained 10% FCS. MycER fusion protein is induced by addition of 0.2 μM 4-OH-tamoxifen (4-OHT) to the media. Protein synthesis is blocked by addition of 10 μM cycloheximide (CHX) to the media 30 min prior to 4-OHT induction.

**Immunofluorescent Microscopy**—CO2-treated cells were grown in high glucose DMEM to 50–70% confluence on glass coverslips and were transiently transfected with 2 μg/ml DNA and 100 μM chloroquine in DEAE-dextran/high glucose DMEM. After 5 h at 37°C, cells were given fresh media and were incubated at 37°C for 48 h prior to antibody staining. Cells were fixed in 3.5% paraformaldehyde/phosphate-buffered saline for 8 min and then permeabilized in 0.1% Nonidet P-40/phosphate-buffered saline for 15 min. Fixed cells were blocked for ≥20 min in 10% fetal calf serum/phosphate-buffered saline before staining with antibodies diluted in the same. Polyclonal peptide antibodies to JPO1 were diluted 1:200 while a secondary antibody was a tetramethylrhodamine B isothiocyanate-labeled goat anti-rabbit antibody diluted 1:200. Coverslips were then mounted onto glass microscope slides with glycerol-containing n-propyl gallate and fixed in place with clear fingernail polish. Slides were visualized by confocal fluorescent microscopy.

**JPO1 Expression Vectors**—Full-length JPO1 cDNA was created by ligating the 5’ end from one clone identified in the library screen to the 3’ end from another clone at the unique EcoRI site internal to JPO1. This full-length JPO1 cDNA was subcloned into the NotI sites of a pSG5 expression plasmid (50) with a modified polylinker site. Expression of JPO1 in pSG5 is from the early SV40 promoter. The entire 1.2-kb JPO1 coding region was amplified with primers containing NotI sites on the ends for ligation into the expression plasmid pBabePuro. PCR-amplified JPO1 sequence was confirmed by sequencing. Expression in pBabePuro is from the retroviral long terminal repeat promoter.

**Stable JPO1 Cell Lines**—Rat1a-derived cells were transfected by lipofection (Life Technologies, Inc.). Cells transfected with pSG5 or MLV expression plasmids were co-transfected with an empty selection marker, pBabePuro or pSVneo, at a ratio of 1:10 (selection plasmid: expression plasmid). Cells were moved into selection antibiotics 48 h after transfection. CB33 B lymphoblast-derived cells were transduced 4–18 h in the presence of 8 μg/ml Polybrene with control pBabePuro retrovirus or JPO1-expressing retrovirus produced from PG13 cells. Cells were moved to puromycin selection 1–2 days after retroviral transduction. Rat1a-transfected cells and CB33 transfected cells were selected in either 750 μg/ml puromycin or 400 μg/ml G418.

**Soft Agar and Methylicellulose Colony Formation Assays**—Soft agar assays were performed by seeding 1.2 × 10^6 cells in a layer of 0.4% agarose/DMEM over a layer of 0.7% agarose/DMEM in a 100-mm plate. A top layer of liquid DMEM was added and changed every 3rd day. Colony formation was assayed at day 14 by light microscopy or by using a colony counting system that recognizes colonies and the UV LabWorks colony counting program (UVP, Upland, CA). Methylcellulose assays were performed by adding 10^5 cells and 2.7-ml supplement (1× IMDM, 26.6% fetal calf serum) to 2.2 ml of a 3% methylcellulose/IMDM mixture and vortexing vigorously. Bubbles were allowed to settle for 5–10 min, and 1 ml of the mixture was added per 35-mm non-treated suspension plate (2000 cells/plate) from a syringe with a 16-gauge needle. Individual 35-mm plates were placed in a larger dish with an extra 35-mm plate containing sterile water for the purpose of humidification. Plates were maintained in a humidified 37°C incubator for 10 days prior to assaying colony formation.

**Mouse Tumorigenic Assays**—5 × 10^5 cells were injected subcutaneously into the right flank of male homozygous nude mice, 4–6 weeks of age from Taconic, Germantown, NY. Tumors were established and weighed at 6 weeks after injection or until tumor mass reaches 1500 mg. Experiments were approved by The Johns Hopkins School of Medicine Animal Care and Use Committee.

**RESULTS**

**Cloning of Human JPO1—**JPO1 was identified as a differentially expressed gene in an RDA of non-adherent Rat1a versus Rat1a-Myc fibroblasts (36). The differentially expressed rat clone had significant homology to human EST H59390. Full-length human JPO1 cDNA was cloned from a human spleen cDNA library using this EST as a probe. The longest of the clones identified in the library screen was 2.4 kb in length. Primer extension was performed on the 5’ end to determine that full-length JPO1 transcript is 2.5 kb (data not shown). Attempts to clone the additional sequence by 5’-rapid amplification of cDNA ends were unsuccessful, so the 5’ sequence was determined by sequencing a P1 clone that contained genomic JPO1 DNA. The composite sequence of full-length JPO1 cDNA is shown in Fig. 1A. Theoretical translation of the cDNA predicts a 371-amino acid protein that contains a putative leucine zipper and a cysteine-rich region (Fig. 1B). A BLASTP search of the non-redundant data base revealed no known proteins with significant homology to JPO1. The JPO1 GenBank™ accession number isAY029179.

**JPO1 Expression Correlates with c-Myc Expression**—Rat1a cells are an immortalized, non-transformed line. With enforced c-Myc expression, these cells are able to grow non-adherently in semi-solid media and undergo tumorigenesis in immunocompromised mice. The effect of cellular adherence on JPO1 expression levels was examined by Northern blot analysis of mRNA collected from both adherent and non-adherent Rat1a and Rat1a-Myc cell lines (Fig. 2A). We chose vimentin as an internal standard for mRNA sample loading, because we found that c-Myc does not affect vimentin levels in Rat1 fibroblasts (36). JPO1 transcript levels are higher in attached cells as compared
with unattached cells. To confirm further the differences in JPO1 expression in non-adherent cells, we used the more sensitive RNase protection assay that revealed a 20-fold higher JPO1 transcript level in non-adherent Rat1a-Myc fibroblasts than in the non-adherent Rat1a fibroblasts (Fig. 2B). Hence, enforced c-Myc expression increases the levels of JPO1 in both attached and unattached cells, although the differential expression is magnified in unattached cells.

JPO1 transcript levels were compared with c-Myc levels in additional cell lines. The TGR (myc+) cell line is a derivative of the Rat1a cell line. HO15 (myc−) cells were created by targeted deletion of both c-myc gene copies from TGR (myc+) cells resulting in a severe cell cycle delay (47). mRNA analyses of the myc−/− cells have been hampered by a global change in transcript levels such that comparison with wild-type cells has been performed on the basis of total RNA as reflected by 28 S ribosomal RNA (47). Hence, for load control, we used 28 S ribosomal RNA. Logarithmically growing HO15

**Fig. 1. JPO1 cDNA and amino acid sequence.** A, full-length human JPO1 cDNA was cloned from a human spleen cDNA library (CLONTECH). The coding region is indicated in **bold**. B, JPO1 cDNA predicts a 371-amino acid protein. The leucine/isoleucine in the putative leucine zipper are **underlined**. The cysteine-rich region is **shaded**.
Novel c-Myc Target JPO1

JPO1 Is c-Myc-responsive—A Myc-inducible cell line was used to determine whether JPO1 levels were elevated directly as a consequence of c-Myc induction. Rat1a-MT-Myc cells, in which c-Myc is under the control of the metallothionein promoter, were induced with 150 μM zinc. The c-Myc protein is elevated within 1 h and peaks maximally from 2 to 6 h post-zinc induction (Fig. 3A). RNA collected from zinc-induced cells over the same time course shows that JPO1 expression, as assayed by RNase protection assay, is elevated 2.5-fold at 6 h (Fig. 3A), adding additional support for the hypothesis that JPO1 is a c-Myc target.

To determine whether JPO1 is a direct or an indirect target of c-Myc, the Rat1a-MycER system was utilized. These Rat1 cells are stably transfected with a chimeric fusion protein composed of c-Myc and the hormone binding domain of the estrogen receptor (49). This MycER fusion protein remains sequestered in the cytoplasm by heat shock proteins except upon exposure to the estrogen analogue 4-OHT. 4-OHT binds the ER portion of the fusion protein, initiating its translocation into the nucleus where it then binds to and transactivates endogenous c-Myc target loci. Pretreatment of MycER cells with the protein synthesis inhibitor, CHX, allows the identification of only those genes whose expression depends directly on c-Myc transactivation and not on an intermediate step that requires protein synthesis. Thus by measuring mRNA induction in cells treated with only 4-OHT and in cells treated with both 4-OHT and CHX, one can distinguish between direct and indirect targets.

JPO1 transcript levels were measured in MycER cells over a 6-h time course after treatment with either CHX alone, 4-OHT alone, or both (Fig. 3B). We chose HuPO (3B4) as a loading control, because we and others (46) have found that HuPO mRNA levels do not vary with stimulation of MycER cells. In 4-OHT-treated cells, JPO1 levels rose steadily to 6 h, showing nearly a 3-fold induction. Cells treated with CHX alone show no JPO1 induction; instead JPO1 levels dropped by 6 h. Cells treated with both 4-OHT and CHX showed approximately a 1.7-fold induction in JPO1 transcript levels at 4 h that is not sustained at 6 h perhaps due to the inhibitory effect seen with CHX alone. These results indicate that JPO1 behaves as a direct c-Myc target gene.

JPO1 Expression in Human Tissues—The expression levels of JPO1 in various human tissues were assayed by probing a normalized human RNA Master Blot (CLONTECH). JPO1 levels were found to be highest in small intestine and thymus (Fig. 4). Additionally, JPO1 was highly expressed in fetal thymus, fetal lung, colon, stomach, appendix, and testis. In contrast to the high expression level seen in thymus, JPO1 levels were low in bone marrow, spleen, lymph node, and peripheral leukocytes.

JPO1 Localizes to Chromosome 2q31—Two independent P1 clones containing JPO1 genomic DNA were used as probes for fluorescent in situ hybridization to map the chromosomal localization of JPO1. JPO1 maps to chromosome 2q31 as the center of the signals by proportional length (data not shown). Mapping data from the UniGene cluster (Hs. 333893; previously assigned to Hs. 47378) that corresponds to JPO1 has subsequently confirmed this map location.

JPO1 Is a 47-kDa Nuclear Protein—Western blot analysis of JPO1-transfected COS7 lysates indicates that JPO1 is a 47-kDa protein (data not shown). The subcellular localization of JPO1 was determined by immunofluorescent microscopy of transiently transfected COS7 cells. Polyclonal peptide antibodies to JPO1 detected strong nuclear staining in JPO1-transfected COS7 cells. Two patterns of nuclear staining are seen. The majority of JPO1-transfected cells showed diffuse nuclear staining exclusive of the nucleolus (Fig. 5F). Additional cells had a punctate nuclear pattern (Fig. 5D) that did not co-localize with proliferating cell nuclear antigen (data not shown). Empty vector transfected cells (Fig. 5B) and untransfected cells showed punctate nuclear staining, which is also seen with pre-immune sera (data not shown) and is dependent on the method of fixation used.

CB33, CB33Myc, as well as the Burkitt lymphoma cell lines were analyzed by RNase protection assay for JPO1 expression. JPO1 was 20-fold overexpressed in Rat1a-Myc cells. JPO1 levels were measured by Northern blot for JPO1 expression, as assayed by RNase protection assay. RNase protection assay, is elevated 2.5-fold at 6 h (Fig. 3A) and unattached Rat1a or Rat1a-Myc cells (data not shown).

Western blot in the B lymphoid cell lines compared with Myc expression levels by loading control. D, JPO1 expression was compared with Myc expression levels by Western blot in the B lymphoid cell lines CB33, CB33Myc, as well as the Burkitt’s cell lines Ramos, Thomas, and Wynn.

CB33 overexpressing c-Myc (CB33-Myc), three Burkitt lymphoma cell lines (Fig. 2) that are wild-type TGR (myc ++/+ ), HET (myc ++/− ), HO15 (myc −/− ), HO15 (myc −/− ) + MLV, and HO15 (myc −/− ) + Myc. 28S was used as a loading control. D, JPO1 expression was compared with Myc expression levels by Western blot in the B lymphoid cell lines CB33, CB33-Myc, as well as the Burkitt’s cell lines Ramos, Thomas, and Wynn.

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Phenotypic Analysis of JPO1 Overexpressing Cell Lines—Since JPO1 expression is highly elevated in Myc-transformed cells, we sought to determine whether JPO1 overexpression is sufficient to induce Myc-related phenotypes. Rat1a cells were transfected with human JPO1 cDNA expressed from the early SV40 promoter in the pSG5 plasmid vector. CB33 human B lymphoid cells were transduced with pBabe-JPO1 retrovirus that expresses JPO1 from the retroviral long terminal repeat. Two independent pools of stable Rat1a-JPO1 cells were generated with high levels of exogenous JPO1 expression (Fig. 6A), and one stable CB33-JPO1 pooled cell line was identified that overexpresses JPO1 3-fold (Fig. 6D).

Overexpression of JPO1 did not alter cellular growth rates in either Rat1a or CB33 cells (data not shown). Involvement of JPO1 in the predisposition of cells to apoptosis was studied in Rat1a-JPO1 cells. Neither growth in 0.1% serum nor growth in low glucose led to any change in levels of apoptosis as compared with the parental control (data not shown).

The ability of Rat1a cells to grow anchorage-independently and the clonogenicity of CB33 lymphoid cells were tested in the soft agar colony growth assay. Rat1a-Myc cells robustly form colonies in soft agar. Rat1a-JPO1 cells also form colonies in this assay (Fig. 6B), averaging about 1/4 the number of colonies that Rat1a-Myc cells form (Fig. 6C). It should be noted that Rat1a colony formation induced by JPO1 is variable as compared with c-Myc, which consistently, robustly induces colony formation. The average size of JPO1 colonies is slightly smaller than that of c-Myc colonies, as none of the pSG5 or JPO1 colonies exceed 200 μm within the 2-week assay period, whereas 4% of the colonies formed by Rat1a-Myc cells do.

To test further the ability of JPO1 to enhance clonogenicity of human lymphoblastoid cells, CB33-pBabe (control), CB33-JPO1, and CB33-Myc cells were tested for colony formation in methylcellulose. As was seen for Rat1a cells, overexpression of either JPO1 or c-Myc increased colony formation in CB33 cells (Fig. 6, E and F). In contrast to the diminished activity of JPO1 in Rat1a cells, JPO1 expression in CB33 cells allows for colony formation intermediate to that of CB33 or CB33-Myc cells.

Although JPO1 induces anchorage-independent growth, this activity is insufficient for tumorigenesis as determined in the nude mouse xenograft model. All 10 mice injected with Rat1a-Myc cells developed tumors with masses of 1490 ± 199 mg (mean ± S.D.). By contrast, only 4 mice injected with Rat1a-JPO1 cells form palpable masses (119 ± 47 mg), which are neither larger nor more frequently found than control Rat1a-pSG5 cells. Although JPO1 induces clonogenicity of CB33 cells and a diminished anchorage-independent pheno-
type in Rat1a cells, JPO1 overexpression is insufficient for tumorigenicity. These observations suggest that other c-Myc target genes in addition to JPO1 are required for tumorigenicity induced by c-Myc.

**Novel c-Myc Target JPO1**

**FIG. 5.** JPO1 is a nuclear protein. COS7 cells were transiently transfected with either pSG5 or pSG5JPO1. Cells were fixed and stained with JPO1 polyclonal peptide antibodies and then with tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit secondary antibodies. Cells were visualized by confocal microscopy. pSG5 transfected cells by phase contrast (A) or by immunofluorescence (B), and pSG5JPO1 transfected cells by phase contrast (C and E) or by immunofluorescence (D and F).

**FIG. 6.** JPO1-overexpressing cells demonstrate anchorage-independent growth. A, two independent pools of Rat1a-JPO1-overexpressing cells were created. JPO1 expression was determined by Western blot, which only detects human JPO1 and not endogenous rat JPO1. B, Rat1a-JPO1 cells form colonies when grown in soft agar. Photomicrographs of selected fields are shown. C, the clonogenic efficiency of Rat1a-JPO1 cells is about one-fourth that of Rat1a-Myc cells. Cells were plated in soft agarose, and photomicrographs (×10) were taken after 2 weeks for quantitation of colonies. The results of 16 independent experiments were averaged with S.E. shown. D, CB33 cells were infected with pBabeJPO1, and JPO1 expression was verified by Western blot. CRB, cross-reacting band. E and F, CB33-JPO1 cells demonstrate increased clonogenicity in methylcellulose as compared with CB33 and CB33-Myc. E, cells were seeded in methylcellulose, and dark field images of whole plates were taken after 2 weeks. F, the results obtained by a UVP imaging system of two independent experiments were averaged with S.E. shown.

JPO1 Complements the Myc Mutant W135E in Soft Agar Transformation Assays—To determine further the role JPO1 plays in Myc-mediated transformation of Rat1a cells, we studied a Myc mutant (W135E) that retains transcriptional
activation potential but neither represses Inr-dependent transcription nor efficiently transforms Rat1a cells (51). W135E retains the ability to increase transcription of some Myc target genes (LDH-A and rcl) but is unable to increase the transcription of other target genes (ODC) to the same level as c-Myc. Like ODC, the expression of JPO1 is severely impaired in W135E cells (Fig. 7A). This observation led us to question whether ectopically expressed JPO1 could restore the transformation efficiency of W135E cells.

The Rat1a-JPO1 line and a control Rat1a-pSG5 (empty vector) pooled cell line were stably transfected with empty MLV vector, Myc, or W135E (Fig. 7B). The soft agar transformation efficiency of these cells was assayed. The Rat1a-JPO1-MLV cell line has diminished soft agar transforming capacity as compared with c-Myc-overexpressing (pSG5 + Myc) cells. In these experiments, the control pSG5 and MLV-transfected Rat1a cells display a high level of background colonies. However, co-expression of either Myc or W135E with JPO1 significantly increases the efficiency of colony formation as compared with control cells that express either Myc alone or W135E alone (Fig. 7C). The transformation efficiency of JPO1 and W135E was comparable with that of Myc alone, suggesting the possibility that JPO1 activity can in fact completely complement the defect in W135E.

**DISCUSSION**

The identification of *bona fide* c-Myc target genes that are relevant to Myc biology remains a formidable challenge. This challenge is further heightened by the advent of microarray analysis of gene expression, which has already generated hundreds of putative c-Myc-responsive genes. Whether the vast

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2 L. A. Lee, unpublished data.
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majority of these putative c-Myc-responsive genes play a role in Myc biology remains unknown. Before the practical availability of microarray analysis, which only identifies differential expression of abundantly expressed genes represented on the microarrays, we sought to determine c-Myc target genes that may participate in the anchorage-independent growth phenotype conferred by overexpression of c-Myc in Rat1a cells. A novel gene, JPO1, was identified as a highly differentially expressed clone in a representational difference analysis of non-adenherent Rat1a versus Rat1a-Myc cells. In this study, we demonstrate that JPO1 expression correlates extremely well with c-Myc expression in numerous cell systems. Genetic evidence for JPO1 as a Myc target gene is provided by the observation that myc null cells have significantly reduced levels of JPO1 that are restored upon exogenous expression of c-Myc. Finally, JPO1 behaves as a direct c-Myc target in the MycER system. In aggregate, results from several approaches suggest that JPO1 is a direct c-Myc target gene.

In normal tissues, JPO1 is most highly expressed in thymus and small intestine. Intriguingly, JPO1 expression is very low in spleen, bone marrow, lymph node, and peripheral leukocytes, suggesting that high levels of JPO1 expression may be restricted to immature T cells and that lowering the levels of JPO1 expression may be necessary for the development of mature lymphocytes. JPO1 localizes to chromosome 2q31 by fluorescent in situ hybridization, and there are several citations in the literature of chromosomal abnormalities at 2q31. These abnormalities were reported in two cases of non-Hodgkin’s lymphoma (52, 53), one case of chronic myeloid leukemia (54), and one case of adult T cell leukemia (55). Whether these translocations affect JPO1 expression remains to be established.

Polyclonal peptide antibodies directed against human JPO1 detected a 47-kDa band. The predicted molecular mass of JPO1 was 42.5-kDa, suggesting the possibility of post-translational modification of the protein or altered migration in SDS-PAGE due to amino acid composition. The JPO1 protein has limited homology to a number of plant proteins through a cysteine-rich region. Although the cysteine-rich region does not conform to any known zinc finger motif, several of the plant proteins are annotated as DNA-binding proteins. These include early nodulin promoter-binding protein (56) and early nodulin promoter-binding protein-like proteins from multiple organisms and c-module-binding protein that binds the AT-rich DNA of the c-module in a Dictostylium repetitive element (57). These homologies suggest that this cysteine-rich region may play a role in DNA binding, and hence JPO1 may be a DNA-binding protein.

Importantly, immunofluorescent microscopic results indicate that JPO1 is a nuclear protein.

As alluded to previously, we observed a strong correlation between c-Myc and JPO1 expression in a variety of human cancer cell lines. Expression of JPO1 was assayed by Western blot in several Burkitt’s lymphoma cell lines and in a series of breast cancer cell lines. JPO1 was found to be overexpressed in these tumor cell lines and to correlate extremely well with c-Myc expression. Because c-Myc overexpression is associated with many primary human tumors, it will be instructive to determine in future studies whether JPO1 overexpression is also found in these primary tumors.

The correlation of JPO1 and c-Myc expression in cancer cell lines suggests the possibility that JPO1 may participate in Myc-mediated phenotypes. Phenotypic analysis of Rat1a–JPO1 cells indicates that JPO1 neither affects cellular growth rates nor induces susceptibility to apoptotic stimuli. However, JPO1 is involved in anchorage-independent growth as was shown by the ability of JPO1-overexpressing cells to form colonies either in soft agar or methyloseullose assays, mimicking the ability of tumor cells to grow independent of anchorage. It is notable, however, that overexpression of JPO1 is insufficient for the tumorigenesis of Rat1a cells as determined by the nude mouse xenograft model. JPO1 complements a Myc mutant (W135E) that has lost its ability to efficiently form colonies in soft agar assays, confirming and emphasizing the importance of JPO1 in anchorage-independent growth.

Although transcriptional repression is an activity associated with Myc Box II, mutation in this region also results in loss of transactivation of specific target genes. For example, the Myc Box II deletion mutant (residues 106–143) does not transactivate ODC (58). Likewise, the Myc Box II mutant W135E does not activate JPO1 or ODC, whereas activation of LDH-A or rcl is preserved.2 It is notable that the Myc Box II region is required for the recruitment of the TRRAP-histone acetylase complex and hence is required for transactivation, perhaps, of genes that require histone acetylation for their transcription (59–61). JPO1 may be such a gene that requires histone acetylation for its expression. As such, complementation between JPO1 and the Myc Box II mutant W135E in transformation provides genetic evidence for JPO1 as a functional target gene of c-Myc.

Here we describe the cloning and characterization of a novel c-Myc target, JPO1, and its involvement in anchorage-independent growth. The identification of c-Myc targets and the association of these target genes with discrete Myc-related phenotypes allow us to construct a model by which Myc influences numerous cellular pathways. The involvement of c-Myc targets in distinct subsets of Myc-related phenotypes underscores the notion that independent pathways interact to form a network leading from c-Myc ultimately to tumorigenesis with Myc targets sitting at nodal points within the network.

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