Analysis of gene expression during *myc* oncogene-induced lymphomagenesis in the bursa of Fabricius

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The transcriptional effects of deregulated *myc* gene overexpression are implicated in tumorigenesis in a spectrum of experimental and naturally occurring neoplasms. In follicles of the chicken bursa of Fabricius, *myc* induction of B-cell neoplasia requires a target cell population present during early bursal development and progresses through preneoplastic transformed follicles to metastatic lymphomas. We developed a chicken immune system cDNA microarray to analyze broad changes in gene expression that occur during normal embryonic B-cell development and during *myc*-induced neoplastic transformation in the bursa. The number of mRNAs showing at least 3-fold change was greater during *myc*-induced lymphomagenesis than during normal development, and hierarchical cluster analysis of expression patterns revealed that levels of several hundred mRNAs varied in concert with levels of *myc* overexpression. A set of 41 mRNAs were most consistently elevated in *myc*-overexpressing preneoplastic and neoplastic cells, most involved in processes thought to be subject to regulation by Myc. The mRNAs for another cluster of genes were overexpressed in neoplasia independent of *myc* expression level, including a small subset with the expression signature of embryonic bursal lymphocytes. Overexpression of *myc*, and some of the genes overexpressed with *myc*, may be important for generation of preneoplastic transformed follicles. However, expression profiles of late metastatic tumors showed a large variation in concert with *myc* expression levels, and some showed minimal *myc* overexpression. Therefore, high-level *myc* overexpression may be more important in the early induction of these lymphomas than in maintenance of late-stage metastases.

The advent of DNA microarray technology for assessment of genome-wide changes in gene expression provides opportunities for more comprehensive exploration of complex processes such as neoplastic change (1, 2). Such an opportunity is presented by the transcriptional effects of deregulated c-*myc* gene overexpression, which are implicated in tumorigenesis in a spectrum of experimental and naturally occurring neoplasms (reviewed in refs. 3–5). Experimental induction of B-cell lymphomas in the bursa of Fabricius of chickens provides one model tumor system in which deregulated overexpression of c-*myc* drives an apparently multistaged neoplastic process in which the normal target cell population and a preneoplastic cell population, as well as fully developed clonal tumors and derivative cell lines, have been identified and can be conveniently obtained for analysis (reviewed in ref. 6). In this system, tumor induction by c-*myc* requires a target cell population present only during embryonic bursal development (7, 8) and produces, as its initial histological manifestation, multiple preneoplastic lesions called transformed follicles (TF) (9–11). The cells comprising TF are a monomorphic population of IgM-positive lymphoblasts that display surface antigens of early bursal cells and that retain stem cell function (8, 10). They differ from the developmentally regulated stem cell population of normal bursal follicles, which disappears shortly after hatching, by persisting in the posthatching bursa and by progressing to form invasive bursal lymphomas that metastasize to liver and other organs. Immortalized cell lines have been generated from *myc*-induced bursal lymphomas, perhaps the best characterized of which is DT-40 (12). DT-40 cells have the property, rare among established vertebrate cell lines, of an efficiency of homologous recombination approaching that of illegitimate recombination (13), making them useful for targeted gene deletion and replacement experiments.

To gain new insights into the genes and genetic networks important in mediating neoplastic change in this model system, we developed a chicken immune system glass slide microarray based on DT-40 and supplemental chicken T-cell and B-cell cDNAs. Analysis of mRNA expression was carried out at several stages of normal embryonic bursal development and of *myc*-induced preneoplastic TF, metastatic lymphomas, and the DT-40 cell line. In each case, comparison was made to expression levels in the normal posthatching bursa where, despite intense proliferative activity in the lymphoid population of normal follicles, endogenous c-*myc* expression is relatively low (14).

Software for cluster analysis (15, 16) was used to identify genes showing similar patterns of expression. These studies suggest that high levels of *myc* overexpression, and consequent broad changes in gene expression, may be important for TF induction but are not consistently required for maintenance of late metastatic disease. Genes whose level of expression in TF and metastatic tumors correlated positively with the level of *myc*-overexpression were identified. This analysis suggests that the effects of *myc* on cellular growth and energy controls may be a predominant mechanism in *myc*-induced neoplasia.

Materials and Methods

cDNA Libraries, DT-40 Expressed Sequence Tag Library, and the Chicken Immune System Glass Slide cDNA Microarray. The microarray constructed for these studies used chicken lymphocyte cDNAs from three different libraries. A cDNA library containing about 1.6×10^6 recombinants, with an average insert size of about 1.5 kilobase pairs, was prepared from DT-40 poly(A)$^+$ RNA by using mixed oligo dT and random primers and cloned into the *Eco*RI site of lambda ZAP II (Stratagene). To reduce

Abbreviations: TF, transformed bursal follicle; HB1, recovered avian myelocytomatosis virus strain MC-29-HB1; SOM, self-organizing map; NB, normal 2-week posthatching bursa.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (for accession nos., see Table 2, which is published as supplemental data on the PNAS web site, www.pnas.org).

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redundancy and unwanted avian retroviral RNAs, the library was subtracted with biotinylated poly(A)$^+$ RNA from normal 2-week posthatching bursa (NB) and avian leukosis virus, as previously described (17). Cultures of individual plasmid clones (in pBluescript SK−, Stratagene) of this subtracted DT-40 library, called DT40subNB, were archived in a 96-well format. The cDNA inserts were amplified from replica plate cultures by PCR, and 1,800 verified products were used both for single-pass DNA sequencing and for robot-assisted spotting onto polylysine-coated glass slides, as previously described (18, 19). Database searching provided gene identifications for about 68% of successfully sequenced DT40subNB cDNAs. Sequence homology cluster analysis (CAP-3) of the library indicated about 55% distinct sequences. Clones with expression patterns of interest in the array experiments, but which were not successfully sequenced in the initial single-pass attempt, were selectively resequenced and gene identifications obtained by database searches. An annotation file with GenBank accession numbers for DT40subNB clones on the array is published as Table 2 in the supplemental data on the PNAS web site, www.pnas.org.

Additionally 1,200 unique clones from a sequenced gene-identified chicken activated T-cell cDNA library previously prepared by one of us (J.B.)[¶] were added to the array (20). Finally, 50 selected nonoverlapping expressed sequence tagged clones from a normal 2-week posthatching bursal library, DKFZ426, previously sequenced and gene-identified by J.-M. Buerstedde (21), were supplied by the Resource Center of the German Human Genome Project and added to the array. The completed array contained 3,011 chicken lymphocyte cDNA spots representing about 2,200 different genes.

Retroviral *myc*-Transduction and RNA Preparation from Tissues and Cells. The reference RNA for all experiments was prepared from normal 2-week posthatching bursa from inbred Line SC white leghorn chickens (Hyline International, Dallas, IA). Suspensions of follicular lymphocytes were prepared as described (8, 10), and total RNA extracted from aliquots of 4×10^7 cells with RNAzol (Tel-Test, Friendswood, TX). As a control for contamination by bursal stromal cells, RNA was extracted from bursas in which the lymphoid population was selectively ablated by treatment with cyclophosphamide (22).

Bursal neoplasia was induced with a replication-defective Moloney leukemia virus- based retroviral vector (23) expressing the *myc*-oncogene of the recovered avian myelocytomatosis virus strain MC-29-HB1 (HB1), L*myc*SN, as previously described (24). Briefly, embryonic bursal cells were cocultivated overnight in L*myc*SN producing monolayers and injected into cyclophosphamide-treated day 18 embryos to repopulate ablated follicles. At 4 weeks, posthatching successfully transduced, reconstituted follicles had TF morphology. By transplanting these cells into cyclophosphamide-ablated secondary recipients, essentially pure populations of TF were generated (8, 24), from which RNA was prepared as from normal bursa. Large metastatic lymphomas appeared beginning 8 weeks after either primary or secondary transplantation. Hepatic tumor masses were dissected away from surrounding normal liver and RNA extracted. RNA was also extracted from normal liver as a control for contaminating liver cells.

Generation of Fluorescent cDNA Probes, Hybridization, and Data Processing. Poly(A)$^+$ RNA was prepared from total RNA by using a Qiagen (Chatsworth, CA) Oligotex mRNA kit and used as template to generate cDNA probes labeled with Cyanine dye-conjugated (Cy3 or Cy5) dUTP (Amersham Pharmacia). Labeling reactions contained 2 μg of Poly(A)$^+$ RNA, 4 μg Oligo(dT)$_{18}$, and 3 μg random hexamer primers in 30 μl of 50 mM Tris·HCl, pH 7.5/40 mM KCl/6 mM $MgCl_2$/10 mM DTT/0.4 mM dATP, dGTP, dCTP/0.15 mM TTP/0.1 mM Cy-dye-labeled dUTP/400 units of Superscript II reverse transcriptase (GIBCO/BRL). Reactions were incubated at 42°C for 1.5 h, diluted to 500 of 10 mm Tris, 1 mm EDTA, pH 7.5, concentrated to ≈10 μl in a Microcon-30 filter (Amicon), and filtered through a Millipore UltraFree-MC filter unit. Reference normal bursal probes were labeled with Cy3, and the various experimental test probes with Cy5 (except in two experiments, where the label was reversed to control for any bias intrinsic to the fluorescent labels). In control experiments with CY-3- and CY-5-labeled probes from different preparations of reference normal 2-week bursal RNA, we determined that 3-fold changes in expression were significant. Test and reference probes were combined in 26 μl of 3× SSC (0.45 M NaCl/0.045 M Na citrate, pH 7.0)/10 μg poly adenylate/0.2% SDS, and were competitively hybridized to an arrayed surface under a coverslip for 16 h at 63°C. Slides were washed in 1× SSC and 0.03% SDS (≈1 min), 1× SSC (≈1 min), 0.2× SSC (20 min), and 0.05× SSC (10 min) and dried by centrifugation ($50 \times g$ for 5 min). Fluorescent array images were collected for both Cy3 and Cy5 by using a GenePix 4000A fluorescent scanner (Axon Instruments, Foster City, CA), and image intensity data were extracted and analyzed by using GENEPIX PRO 3.0 microarray analysis software. Normalization of the Cy5 to Cy3 fluorescent signal in each experiment was determined assuming equivalent global hybridization of test and reference probes to genomic chicken DNA.

Results

Analysis of Expression During Normal Bursal Development. Bursal cells were prepared for analysis of expression during normal development. RNA was extracted from day 15 embryos, when hematopoietic bursal migrants have colonized the anlage (25), day 16 when most lymphoid cells are in follicles, day 18 with vigorously dividing embryonic follicular populations, and day 21 initiating posthatching physiology, including disappearance of transplantable stem cell and *myc*-target populations (7, 8). Fig. 1*A* shows that, early in bursal development at embryonic day 15, 196 mRNAs demonstrated 3-fold changes in expression, both increasing and decreasing relative to the posthatching reference. The number of mRNAs showing differences gradually diminished up to hatching at day 21. Fig. 1*B* is a self-organizing map (SOM) (16) that, among the mRNAs demonstrating 3-fold change, identified clusters of cDNAs with similar patterns of change during embryonic development. Gene and clone lists for these clusters are given in Table 3, which is published as supplemental data on the PNAS web site, www.pnas.org. A summary of these data is as follows.

Genes in SOM clusters 1 and 4 (Fig. 1*B*) showed relatively elevated levels of expression in the embryonic day 15 bursal migrants and day 16 follicular cells that then fell during subsequent maturation of the bursa. The mRNAs for a number of T-cell-specific genes, including CD3, T-cell receptor, and granzyme B, were prominent in this set, suggesting a multipotential character of early bursal precursors that is lost during development. Genes in SOM cluster 7 showed relatively high-level expression through embryonic day 18 and then fell by time of hatching. Some mRNAs in this set (but not in the preceding set) were identified as highly expressed in the *myc*-induced bursal neoplasms. Genes in SOM clusters 2, 3, and 6 showed progressive increases in expression from embryonic day 16 as bursal development progressed. Included in this group are genes involved in Ig production and genes promoting apoptotic cell death, such as tumor necrosis factor, *mtd/bok*, and caspase 3. This finding correlates with both the principal function of the bursa in

[¶]Details of the Pat library clones used are found in Array 4 at the University of Delaware Chick EST web site: http://udgenome.ags.udel.chickest.array.htm.

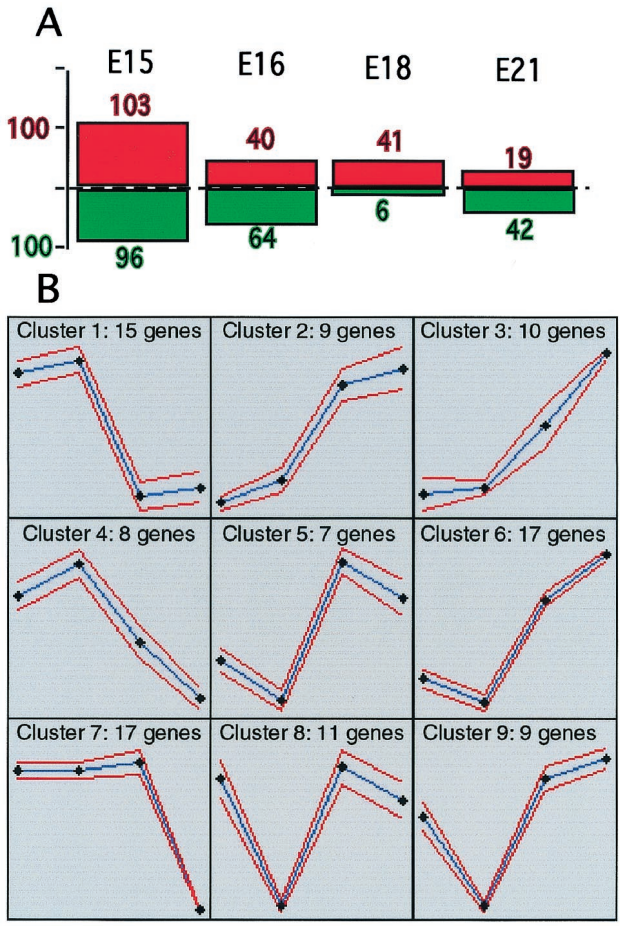

Fig. 1. Gene expression patterns during normal bursal development. (*A*) Chart of numbers of mRNAs showing at least 3-fold increases (red) or decreases (green) at embryonic days 15, 16, 18, and 21, compared with 2 weeks after hatching. (*B*) SOM (17) of 103 mRNAs showing at least 3-fold or greater changes at embryonic day 16 (E16) relative to 2-week posthatching bursa. The expression level of each gene was normalized to have a mean = 0 and SD = 1 across time points. Each cluster exhibits an average pattern (blue) and SD for this average (red). Relative expression levels are shown on the *y* axis and four time points (E15, E16, E18, E21) on the *x* axis.

generating B cells and with the high rates of apoptosis in the fully developed bursa (26, 27).

Gene Expression Correlated with Levels of *myc*-Expression in Preneoplastic TF, Metastatic Lymphomas, and DT-40 Cells. The L*myc*SN vector was used to induce preneoplastic TF and metastatic lymphomas. The long terminal repeat (LTR) enhancer/promoter of the L*myc*SN vector produces modest constitutive overexpression of the *myc* transgene in chicken B-cells at a lower level than that produced from avian retroviral LTRs (24). The level of *myc*-expression in our tissue samples was assessed with three c-*myc* cDNA spots on the array and confirmed by Northern blot hybridization quantitated by PhoshorImager analysis (Molecular Dynamics) (data not shown). We found that among 12 L*myc*SN-transduced samples, *myc* expression levels varied from 1.5- to 8-fold, with only one tumor greater than 20-fold over the level of c-*myc* in reference normal bursal samples. HB1 virus-induced TF (8) in which the same v-*myc* oncogene as in L*myc*SN was overexpressed 38-fold, and the DT-40 bursal lymphoma cell line in which endogenous c-*myc*, driven by a avian leukosis virus LTR was overexpressed 18-fold, were also analyzed. We were therefore able to observe effects of different levels of *myc* expression on global gene expression in these preneoplastic and neoplastic cells.

The number of mRNAs showing 3-fold changes in expression relative to normal 2-week bursa was considerably greater in bursal neoplasia than was observed during normal development. Furthermore, the level of *myc* overexpression appeared to influence the number of genes affected. This result is illustrated in Fig. 2, in which the number of cDNAs showing 3-fold change up or down is plotted against the fold increase in *myc*-expression. Metastatic tumors with relatively low *myc* oncogene expression showed reduced expression of many mRNAs relative to normal bursa. However, at 3-fold or greater increased *myc*-expression, a large but variable number of mRNAs in preneoplastic and neoplastic tissue showed increased expression.

A cluster analysis by the method of Eisen *et al.* (15) was used to identify genes with similar changes in expression. Fig. 3 displays an analysis of about 800 mRNAs showing at least 3-fold changes in expression in at least 6 of the 14 different samples. The cDNAs are displayed on the vertical axis with overexpression represented by red and expression levels below that of normal bursa in green. Different tissue or cell samples are labeled along the top by experimental bird number and identified as TF preparation or metastatic tumors. The normalized ratio of *myc* expression of sample to normal bursa is indicated in parentheses. For the TF samples, most of the mRNAs were overexpressed, particularly in the two samples with the highest levels of *myc* overexpression. Most of these mRNAs were also overexpressed in DT40 cells, but in the metastatic tumors, cDNAs showing overexpression diminished with decreasing levels of *myc* expression, and those showing lower than normal bursal levels of expression increased. With respect to overexpression, the analysis indicated gene clusters A, B, E, and D in order of apparent sensitivity to level of *myc.* Clusters A and B contain genes overexpressed in TF but not in metastatic tumors, suggesting a possible role in preneoplasia but not in end-stage metastases.

Tables 4 and 5, which are published as supplemental data on the PNAS web site, www.pnas.org, are files for generating the data in Fig. 3 and inspecting gene lists for the expression clusters. As a summary, Table 1 lists the gene identities and functions of mRNAs showing the most consistent evidence of increased expression in response to *myc* overexpression. All genes on this list showed 3-fold or greater increases in at least 6 of the 8 samples, with *myc* expression 3-fold or more above that of normal bursa. Tumor-expressed cDNAs that were also expressed highly in liver, but not in bursal samples or DT-40, were eliminated from the analysis as probable liver contaminants, and cDNAs highly expressed in stroma from ablated bursal follicles were also eliminated. This list should include genes up-regulated by both direct and indirect effects of Myc. Some genes previously reported to be direct Myc targets (for reviews, see refs. 5 and 29) were detected. The most consistently up-regulated previously reported *myc* targets (e.g., nucleolin, LDH, enolase, DP-1, Nm-23) are indicated in Table 1.

A small cluster of mRNAs (Cluster C) were overexpressed in most of the preneoplastic and tumor samples (but not in DT40) in a fashion that did not correlate with the level of *myc* expression. Most of these are novel genes, but among those identified were several genes (e.g., folate-binding protein and an ATP-binding mitochondrial transporter) with high-level expression in embryonic bursa through day 18 and which may reflect the differentiation state of these neoplasms. This finding is consistent with an immunohistochemical analysis of antigens expressed during bursal development, which also suggested a maturation block in *myc*-induced TF at about day 18 (29).

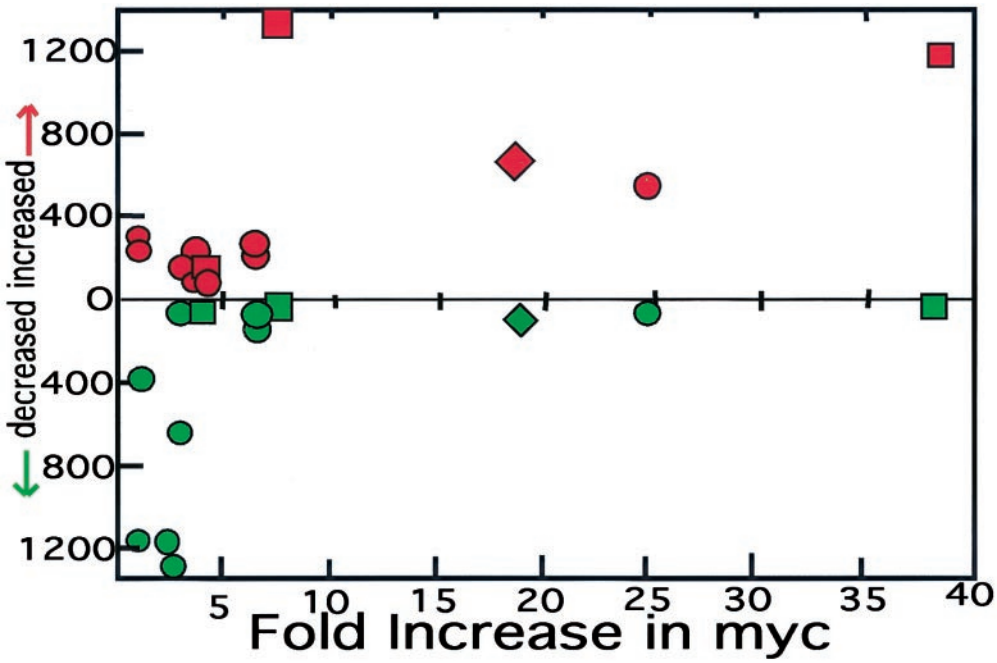

Fig. 2. Relationship of level of *myc*-oncogene expression to change in expression of other mRNAs on the array. The number of mRNAs (*y* axis) showing 3-fold or greater increased (red) or decreased (green) expression, relative to the 2-week NB reference, for three TF preparations (squares), eight metastatic tumors (circles), and the DT40 cell line (diamonds). The fold increase in *myc* expression, determined on the array for each sample, is displayed on the *x* axis.

Discussion

The chicken immune system microarray we describe should be useful for analysis of a variety of avian experimental systems, including targeted gene deletion and replacement in DT40 B-cells (13). Our initial study of the developmental program in bursal lymphocytes demonstrated that a relatively small subset of the about 2,000 different genes included on the array were differentially expressed in embryonic bursal differentiation. In contrast, levels of expression of hundreds of genes appeared to be significantly influenced by the level of *myc*-oncogene overexpression in preneoplastic and neoplastic bursal cells. That such a large fraction of spotted cDNAs detected mRNA expression changes may result in part from the fact that nearly half of the genes on the microarray were obtained from the DT40subNB library providing enrichment for genes with expression enhanced by c-*myc* in a neoplastic cell.

Myc Overexpression Level Influences Gene Expression Profiles During Tumorigenesis. The L*myc*SN retroviral vector we used to express the *myc*-oncogene in the bursa produced levels of transgene expression that varied widely. Although the reason for this variability was not determined, it did afford the opportunity to correlate expression profiles with different level of *myc* overexpression in these clonal (30) neoplasms. Previous microarray analysis of conditional overexpression of Myc-estrogen receptor fusion proteins in cell culture has revealed genes with acute and apparently direct responses to increases in levels of Myc (31–33). The present study of gene expression during *myc*-induced oncogenesis did identify some genes identified by this preceding microarray analysis and other studies to be direct Myc targets (Table 1), along with other genes involved in cell processes thought to be regulated by Myc such as growth, energy metabolism, and cell death (reviewed in ref. 5). Thus, although different in design and intent, the information generated by this analysis seems consistent with and complementary to existing literature. One exception, the cell division cycle, is not well represented in Table 1, perhaps because the B-cell population of normal posthatching bursal follicles is already intensely proliferative.

That several metastatic lymphomas expressed *myc* mRNA at levels that were only marginally, if at all, increased over normal bursa suggests that high-level overexpression of Myc is not required for maintenance of advanced metastases in this model. However, an essential role for constitutive deregulated *myc* expression at a lower level remains quite possible, as demonstrated by the observation that inactivation of a *myc* transgene causes regression of *myc*-induced neoplasms in transgenic mice (34).

It seems more likely that high-level *myc*-oncogene expression, and hence some of the genes that were overexpressed in concert with *myc*, is required for induction of preneoplastic TF. Clusters A and B (Fig. 3) contain genes overexpressed in TF and not in metastases (see supplemental material). These include known *myc* targets such as ornithine decarboxylase, DEAD box RNA helicase, and tumor necrosis factor receptor associated protein-1, which may have a role specific to preneoplasia in this system. In our transplantation model, we had to achieve very high *myc*-expression to induce TF. For example, TF induced by the HB1 virus show very high levels of *myc*-oncogene expression (14), 38-fold above normal bursa levels in the TF sample (R779-TF) we used in this study. When HB1-induced TF are transplanted to ablated bursas in secondary recipients, only full TF morphology is observed (8). L*myc*SN-induced TF in this study showed relatively lower levels of 3- to 7-fold overexpression of the HB1-*myc* transgene. Secondary transplants of these TF, on histological examination, revealed variable degrees of morphological differentiation (data not shown), suggesting that this lower average level of oncogene expression in the polyclonal TF population did not maintain the maturation block in some TF cells. This line of reasoning implies that additional changes in TF cell clones mediate progression to metastasizing lymphomas. When these changes occur, high-level *myc*-oncogene expression and some of the changes in gene expression it induces may be less essential, perhaps to a degree that varies between neoplastic clones.

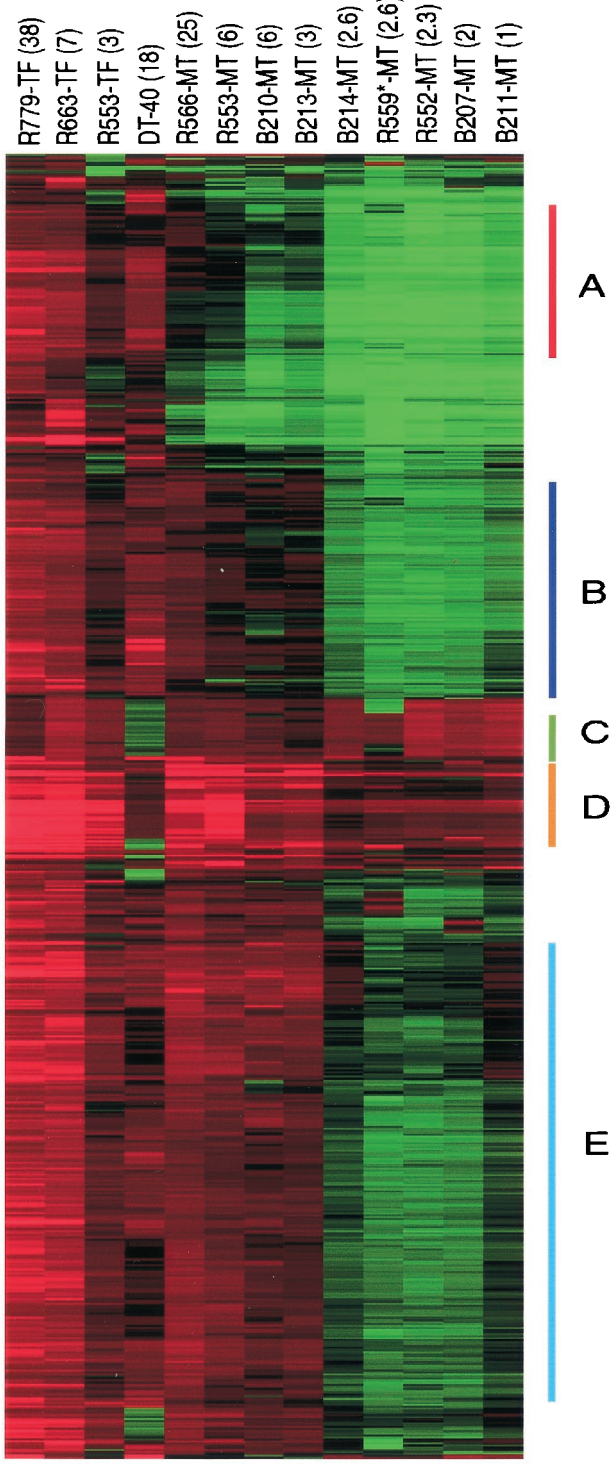

Fig. 3. Gene expression profiles of *myc*-induced neoplastic change in the bursa. mRNAs showing at least a 3-fold change (approximately 800) in expression in at least 6 of 14 experimental samples were clustered. Groups of genes (*y* axis) with similar patterns of expression are indicated by vertical bars (*A*–*E*). DT40, DT40 cell line; MT, metastatic tumor. The fold increase in *myc* expression relative to normal bursa is shown in parentheses.

Table 1. Genes overexpressed in *myc*-overexpressing bursal neoplasia

Homology	Fold increase
Chromatin	
Nucleosome assembly protein-1 (NAP-1)	6.5
Transcription	
Transcription factor E-2 Ig transcription factor	3.5
DP-1 transcription factor*	3.3
Deformed epidermal autoregulatory factor-1 related (NUDR)	4.0
Sterol receptor element-binding protein	4.0
Nucleoli	
Nucleolin*	4.0
Nucleophosmin	3.5
Nucleolar protein 38	3.5
Nucleolar protein p40	3.4
Translation	
Ribosomal proteins S8, 16, 25, L27a	3.1–4.5
Elongation factor 1α	5.0
Elongation factor 1β	5.0
Mitochondria	
Matrix nucl. diphos. Kinase (Nm-23)*	4.5
Heat shock protein (HSP)-60	6.3
Matrix protein P1	5.5
Mitochondrial receptor	4.0
Glycolysis	
Lactate dehydrogenase*	7.0
αEnolase*	4.0
Chaparonins	
Heat shock protein (HSP)-90	7.0
Heat shock protein (HSP)-71	4.0
Cell death	
Tumor necrosis factor receptor 6	3.0
Amyloid precursor	4.3
Tumor suppressor	
PTEN	4.0
Other	
Acetyl CoA-binding protein	4.4
ADP ribosylation factor 5	4.0
L3 phosphoserine phosphatase	8.2
GTP-binding RAB 1A	7.8
Signal peptidase	3.2
B-cell translocation gene (BTG-1)	3.3
Nine novel cDNA sequences	>3.0

*Genes reported to be direct targets of c-*myc*.

The mechanism(s) by which overexpression of *myc*-oncogenes increased expression of the large number of genes observed in this study is also unknown. The promoters of some of the genes in Table 1 are known to have E box-binding sites for Myc/Max heterodimers and to be direct targets for positive transcriptional regulation in experimental systems (5, 28). It is possible that, at abnormally high levels of expression, Myc binds more abundant lower affinity sites and activates transcription from promoters of genes not usually regulated in this manner. Alternatively, Myc is known to interact with proteins that modify chromatin (see ref. 5 for review). At these high levels, Myc might have a widespread effect on chromatin and may increase the probability and/or efficiency of transcription of a large number of genes. One of the mRNAs with consistently elevated expression encodes nucleosome assembly protein 1, a conserved histone-binding protein that promotes nucleosome assembly and may play a role in DNA replication-associated nucleosome assembly (reviewed in ref. 35).

Myc can repress as well as transactivate transcription of target genes (reviewed in ref. 5). In recent DNA microarray experiments testing acute effects of *myc* overexpression in cell culture, more genes were activated than repressed (31–33). In this analysis, we found relatively few genes repressed in samples with

3-fold or greater increases in *myc* mRNA and none that were consistently repressed in most of the samples. That a large fraction of the cDNAs on the array were derived from DT-40 cells that overexpress Myc to very high levels may have limited our opportunity to identify targets of repression in the analysis that could be important in tumorigenesis.

How Does Myc Promote Multistage Tumorigenesis? The direct action of viral Myc on the promoters of a specific set of target genes undoubtedly plays a central role in acute transformation in tissue culture and rapid induction of neoplasms *in vivo* by oncogenic *myc*-transducing viruses. Recent observations of several forms of gross genetic instability induced by *myc* overexpression suggest a second class of mechanisms that could play a role, especially in multistage tumorigenesis (36, 37). However, gross genetic changes of the type described in these reports have not been observed in this bursal lymphoma model system (unpublished observations). The detection in this study of the overexpression of hundreds of genes in apparent response to high levels of *myc*-oncogene expression suggests a third type of mechanism. Specifically, phenotypic change caused by some of the spectrum of overexpressed genes in *myc*-induced preneoplastic cells might be selected to mediate tumor progression. Mutations that fix critical changes might then occur subsequent to this overexpression-driven process and might allow for a lower level of *myc*-oncogene expression in some advanced tumor clones.

Which genes and pathways might be most central in neoplasia mediated by *myc* oncogenes? Inspection of cluster D (Fig. 3) and Table 1 directs attention to the recent discovery of the role of *myc* in regulation of cell growth in fruit flies (38) and mammalian B cells (39). Eleven of the 32 (35%) known genes in Table 1 are involved with nucleolar function, ribosome biogenesis, and protein synthesis. Enlarged and multiple nucleoli are a classical cytological feature of transformed cells. The next most predominant grouping (7 genes, 22%) points to mitochondrial homeostasis and the coordinated processes of glycolysis and cell death (reviewed in ref. 40). These results invite more attention to these facets of cellular regulation in unraveling the role of *myc* in neoplastic as well as normal development.

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1. Brown, P. O. & Botstein, D. (1999) *Nat. Genet.* **21,** 33–37.
2. Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96,** 9212–9217.
3. DePinho, R. A., Schreiber-Agus, N. & Alt, F. W. (1991) *Adv. Cancer Res.* **57,** 1–46.
4. Nesbit, C. E., Tersak, J. M. & Prochownik, E. V. (1999) *Oncogene* **18,** 3004–3016.
5. Grandori, C., Cowley, S. M., James, L. P. & Eisenman, R. N. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 653–699.
6. Neiman, P. E. (1994) *Adv. Immunol.* **56,** 467–484.
7. Maas, H. J. L., Deboer, G. F. & Groenend, J. E. (1982) *Avian Pathol.* **11,** 309–327.
8. Thompson, C. B., Humphries, E. H., Carlson, L. M., Chen, C.-L. H. & Neiman, P. E. (1987) *Cell* **51,** 371–381.
9. Cooper, M. D., Payne, L. N., Dent, P. B., Burmester, B. R. & Good, R. A. (1960) *J. Natl. Cancer Inst.* **41,** 373–389.
10. Neiman, P., Wolf, C., Enrietto, P. J. & Cooper, G. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 222–236.
11. Baba, T. W. & Humphries, E. H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 213–216.
12. Baba, T. W. & Humphries, E. H. (1985) *Virology* **144,** 139–151.
13. Buerstedde, J.-M. & Takeda, S. (1991) *Cell* **67,** 179–188.
14. Neiman, P. E., Gehly, E. B., Carlson, L. M., Cotter, R. C. & Thompson, C. B. (1988) *Curr. Top. Microbiol. Immunol.* **141,** 67–74.
15. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868.
16. Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E. S. & Golub, T. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2907–2912.
17. Sive, H. L. & St. John, T. (1988) *Nucleic Acids Res.* **16,** 10937.
18. Shalon, D., Smith, S. J. & Brown, P. O. (1996) *Genome Res.* **6,** 639–645.
19. DeRisi, J. L., Iyer, V. R. & Brown, P. O. (1997) *Science* **278,** 680–686.
20. Tirunagaru, V. G., Sofer, L., Cui, J. & Burnside, J. (2000) *Genomics* **66,** 144–151.
21. Abdrakhmanov, D., Lodygin, D., Geroth, P., Arakawa, H., Law, A., Plachy, J., Korn, B. & Buerstedde, J.-M. (2000) *Genome Res.* **10,** 2062–2069.
22. Eskola, J. & Toivanen, P. (1974) *Cell. Immunol.* **13,** 459–471.
23. Miller, A. D. & Rosman, G. J. (1989) *BioTechniques* **7,** 980–990.
24. Neiman, P. E., Summers, J., Thomas, S. J., Xuereb, S. & Loring, G. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59,** 509–515.
25. Le Douarin, N. M., Houssaint, E., Jotereau, F. V. & Belo, M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 21701–21705.
26. Neiman, P. E., Thomas, S. J. & Loring, G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5857–5861.
27. Jacobsen, K. A., Paramithiotis, E., Ewert, D. L. & Ratcliffe, M. J. (1996) *Adv. Exp. Med. Biol.* **406,** 155–165.
28. Dang, C. V. (1999) *Mol. Cell. Biol.* **19,** 1–11.
29. Brandvold, K. A., Ewart, D. L., Kent, S. C., Neiman, P. E. & Ruddell, A. (2001) *Oncogene*, in press.
30. Neiman, P., Payne, L. N. & Weiss, R. A. (1980) *J. Virol.* **34,** 178–186.
31. Coller, H. A., Grandori, C., Tamayo, P., Colbert, T., Lander, E. S., Eisenman, R. N. & Golub, T. R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3260–3265.
32. O'Hagan, R. C., Schreiber-Agus, N., Chen, K., David, G., Engelman, J. A., Schwab, R., Alland, L., Thomson, C., Ronning, D. R., Sacchettini, J. C., *et al.* (2000) *Nat. Genet.* **24,** 113–119.
33. Schumacher, M., Kohlhuber, F., Holzel, M., Kaiser, C., Burtscher, H., Jarsch, M., Bornkamm, G. W., Laux, G., Polack, A., Weidle, U. H. & Eick, D. (2001) *Nucleic Acids Res.* **29**, 397–406.
34. Felsher, D. W. & Bishop, J. M. (1999) *Mol. Cell* **4,** 199–207.
35. Krude, T. (1999) *Eur. J. Biochem.* **263,** 1–5.
36. Mai, S., Fluri, M., Siwarski, D. & Huppi, K. (1996) *Chromosome Res.* **4,** 365–371.
37. Felsher, D. W. & Bishop, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3940–3944.
38. Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N. & Gallant, P. (1999) *Cell* **98,** 779–790.
39. Iritani, B. M. & Eisenman, R. N. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13180–13185.
40. Vander Heiden, M. G. & Thompson, C. B. (1999) *Nat. Cell Biol.* **1,** E209–E216.