A Cell Cycle Analysis of Growth-related Genes Expressed during T Lymphocyte Maturation

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Abstract. Fetal liver or bone marrow–derived T lymphocyte precursors undergo extensive, developmentally regulated proliferation in response to inductive signals from the thymic microenvironment. We have used neonatal mouse thymocytes size-separated by centrifugal elutriation to study the cell cycle stage-specific expression of several genes associated with cell proliferation. These include genes involved in the biosynthesis of deoxyribonucleotide precursors, such as dihydrofolate reductase (DHFR), thymidylate synthase (TS), and the M1 and M2 subunits of ribonucleotide reductase, as well as *c-myc*, a cellular oncogene of unknown function. Using nuclear run-on assays, we observed that the transcription rates for these genes, with the exception of TS, are essentially invariant not only throughout the cell cycle in proliferating cells, but also in noncycling (G0) cells. The TS gene showed a transient increase in transcription rate in cells which bordered between a proliferating and nonproliferating status. Studies of an elutriated T cell line, S49.1, yielded similar results, indicating that the process of immortalization has not affected the transcriptional regulation of these genes. Analysis of steady-state mRNA levels using an RNase protection assay demonstrated that the levels of DHFR and TS mRNA accumulate as thymocytes progress through the cell cycle. In contrast, only the M2 subunit of ribonucleotide reductase showed cyclic regulation. Finally, in contrast to cultured cell models, we observed an abrupt fivefold increase in the steady-state level of *c-myc* mRNA in the transition from G1 to S-phase. We conclude from these studies that the transcriptional regulation of specific genes necessary for cellular proliferation is a minor component of the developmental modulation of the thymocyte cell cycle.

RECENT advances using marine invertebrates and genetically well-characterized fungi have demonstrated that the proteins which function in controlling cell cycle progression are highly conserved (for review see 23). Although progress is being made in isolating and characterizing the genes that encode the mammalian versions of these cell cycle regulators, little progress has been made in defining which genes are regulated by the cell cycle, i.e., genes whose expression is inextricably dependent on a particular position in the cell cycle. Not only has progress been slow in this area, it has also been confounded by the lack of a distinction between true cyclic synthesis/degradation and synthesis that is simply growth dependent. This has been particularly true in the case of genes involved in the biosynthesis of nucleotide precursors for DNA replication, such as dihydrofolate reductase (DHFR),[1] thymidylate synthase (TS), and ribonucleotide reductase, which have often been referred to as S-phase–regulated enzymes. By employing the technique of centrifugal elutriation, we have recently shown that expression of the DHFR gene is better understood within the context of growth regulation as opposed to cell cycle regulation (11). In cultured cell lines, conclusions concerning the regulation of expression of particular genes involved in aspects of DNA replication like DHFR, are subject to the caveat that the results may not accurately reflect proliferative processes that occur in an intact animal. Cultured cells may represent variants selected for alterations/deficiencies in gene regulation to compensate for increased demands for a particular enzyme needed for continuous proliferation.

In this study we have investigated this possibility by analyzing the expression of several growth-related genes, namely, DHFR, TS, ribonucleotide reductase, *c-myc*, and histone H4 in neonatal murine thymocytes. Because of the need to expand very small numbers of extrathymically derived precursor cells, the thymus is a site of tremendous cell proliferation. Precursor expansion is developmentally regulated, as evidenced by studies showing that in vivo proliferative capacity is highest among the most immature progeni-

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1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; TS, thymidylate synthase; TCR, T-cell receptor.

tors and decreases with maturation (28). Thus, the thymus provides a good source of cells that are actively dividing in response to developmental cues. The analyses were carried out using nuclear run-on assays to measure rates of transcription and RNase protection assays to quantitate steady-state mRNA levels. We have also compared the transcription rates found in thymocytes to those obtained in an elutriated mouse T-cell line, S49.1. We find that the transcription rates for all the genes tested, with the exception of TS, are invariant across the cell cycle for both thymocytes and the cultured cell line, indicating that the process of immortalization has not grossly affected the transcriptional regulatory patterns for these genes. In addition, we show that in thymocytes that have withdrawn from the cell cycle, these genes are still transcriptionally engaged. The TS gene was the only one that differed in its pattern of expression, showing a transient increase in the rate of transcription in G1/S cells. In contrast, we observed different patterns of mRNA accumulation through the cell cycle for each of the genes. In general, with the exception of *c-myc*, these patterns are in agreement with those observed in cultured cells. The steady-state level of the *c-myc* mRNA displays an abrupt increase during the transition from G1 to S phase that is not observed in cultured cells. We have two major conclusions (*a*) the pattern of expression for the genes involved in the biosynthesis of nucleotide precursors in cultured cells is similar to that found in proliferating thymocytes isolated from intact tissue; and (*b*) changes in mRNA abundance between cycling and noncycling cells for all the genes analyzed, with the exception of TS, seem to depend mainly on posttranscriptional events.

Materials and Methods

Elutriation of Neonatal Mice Thymocytes

Thymuses were removed from 20, 7-d-old B.10 mice, gently teased apart, and cells isolated by repeated washings of the tissue with elutriation medium (PBS containing 1% FBS, 1% glucose, and 30 mM Na_2 EDTA, pH 8.0) through an 85-μm mesh. Lymphocytes were purified by a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Piscataway, NJ) at 18°C. Generally, 2.0–2.7 × 10^8 cells were concentrated to 10 ml, and then loaded into a rotor (model JE-6B; Beckman Instruments Inc., Palo Alto, CA) using a standard chamber and a Masterflex Digi-Staltic pump (Cole Parmer Instruments, Chicago, IL) set at 8 ml/min. Constant rotor speed was maintained at 2,400 rpm at 18°C. A total of 12 250-ml fractions were collected by increasing the pump speed at intervals ranging between 1 and 4 ml/min (see Table I). Elutriation conditions for the mouse T cell line S49.1 (19) were essentially the same. Coulter Channelyzer and flow cytometric analyses of the elutriated fractions were carried out as previously described (11).

Analysis of Cell Surface Markers on Elutriated Thymocytes

Elutriations were carried out as described above except that the fractions were resuspended in cold RPMI medium. Immunofluorescent staining and flow cytometric analyses for CD4, CD8, and TCR (CD3) were carried out as previously described (16).

Determination of the Rate of Transcription by Nuclear Run-on Analysis

Nuclear run-on analyses were carried out as described (14) with the following modifications. Reactions were carried out in Eppendorf tubes using 0.8–5.0 × 10^7 nuclei. After a 30-min pulse with ^{32}P-UTP (3,000 Ci/mmole, Amersham Corp., Arlington Heights, IL), the nuclei were centrifuged in an Eppendorf centrifuge for 15 s, and the RNA was isolated by the single-step acid guanidinium method (5), except that after the first isopropanol precipitation, the RNA was resuspended in 100 μl of STE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl), and the unincorporated nucleotides were removed by centrifugation through Sephadex G-50 spin columns (22). The RNA was precipitated with TCA and subjected to limited NaOH digestion (27). Equal numbers of cpms were hybridized in 0.5 ml to DNA immobilized on Zetabind filters (AMF CUNO, Meriden, CT) using standard conditions (10). All DNA sequences used are in SP6 vectors (Promega Biotech, Madison, WI) with the exception of THY-1 (18) and α-TCR clones (4) and were as follows: pSP65-5′ which contains 310 bp of the 5′-end of the mouse DHFR cDNA (3); pSP65-MycX/S which contains the second exon of the mouse *c-myc* gene (33), pSP64-H4 which contains a 270-bp Hpa II fragment of the mouse histone H4 gene from the plasmid pμ (15) inserted into the Hind III site of pSP65 with Hind III linkers; pSP64-TS which contains the 5′ most 300-bp Pst I–Bam HI fragment from the thymidylate synthase cDNA pMTS-3 (13), pSP64-M1 (35) which contains a 300-bp Pvu II–Bgl II fragment from the M1 ribonucleotide reductase subunit cDNA cloned into the Bam HI–Sma I sites in pSP64; and pSP64-M2 which contains a 250-bp Bam HI–Pst I fragment from the M2 ribonucleotide subunit cDNA (35).

Steady-State RNA Analyses

Total RNA was isolated by the single-step acid guanidinium procedure (5) and 2 μg were analyzed by the RNase protection method (39) using in vitro RNA probes generated from the constructs described above with the modifications previously described (11). RNA amounts were measured on agarose gels. Scanning densitometric analyses of the resulting autoradiograms were carried out on an ultrascan laser densitometer (LKB Instruments, Inc., Gaithersbrg, MD).

Results

Elutriations of Neonatal Mouse Thymocytes

The murine thymic anlagen is first seeded by migratory fetal liver precursors between days 11 and 12 of fetal life. During the next 4–5 d, the newly arrived precursors are expanded by two to three orders of magnitude, and by day 16 some of the progeny express low levels of surface T cell receptor (TCR) and high levels of the CD4 and CD8 coreceptors (12). These TCR^{lo} and $CD4^+8^+$ blast cells then cease proliferating and follow one of three possible developmental fates: the majority die as postmitotic small TCR^{lo} $CD4^+8^+$ cells, but some mature into TCR^{hi} $CD4^+8^-$ or TCR^{hi} $CD4^-8^+$ T cells (16). In late fetal, neonatal, and adult life, the thymus is continuously seeded by very small numbers of bone marrow–derived precursor cells, and these follow the same maturational sequence as the first influx of fetal liver–derived cells (32). Despite new precursor immigration and maturation, the high proportion of cycling cells (80–90%) among early thymocytes drops to 10–15% in the young adult steady-state thymus, with a concomitant increase in cell yield per thymus of over 1,000-fold. Therefore, the optimal age of a mouse appropriate for thymocyte separation by centrifugal elutriation depended on two factors, (*a*) cell yield and (*b*) proportion of these cells still activity dividing. To find that optimum, thymocytes were isolated from neonatal mice between the ages of 5–16 d, and the percentage of cells with volumes >87 μm^3 (the size of a dividing cell) was determined (data not shown). Approximately 65% of the cells met this size criterion at day 7, which was thus the age of cells used for all elutriations.

Fig. 1 shows the DNA histograms obtained from a typical elutriation. Table I presents a summary of the flow rates used to obtain each fraction, the calculated Coulter volume of the cells in that fraction, the percentage of cells in each stage of

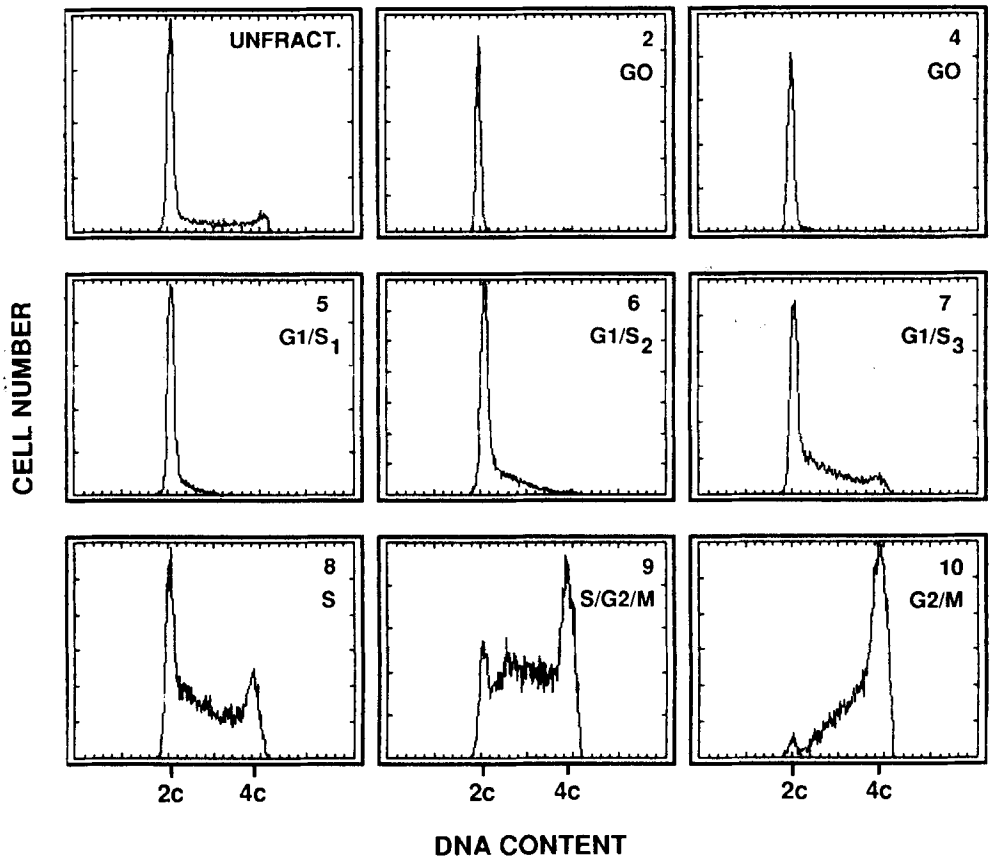

Figure 1. DNA content histograms for elutriated neonatal mouse thymocytes. The unfractionated bimodal profile is representative of a mixture of nondividing and dividing cells. Cells in fraction 2, which represents the smallest $CD4^+8^+$ cells, are entirely nondividing and have a DNA content of 2C. Cells in fractions 4–10 show increasing DNA content (fraction 3 was not used). Cell cycle designations for each fraction as used throughout the text are listed on each histogram and in Table I. Cells prepared in this elutriation represent the one used for RNA isolation and RNase protection in Fig. 3 *A* and Fig. 5. In general, we found thymocyte elutriations to be very reproducible and essentially similar results and values were obtained with the elutriation used in Fig. 3 *B* and Fig. 4.

the cell cycle determined by integrating areas under the histograms in Fig. 1, and the mean DNA content (C-value) for each. The viability of thymocytes after elutriation was determined to be 97–98% by propidium iodide staining (data not shown). Previous functional and phenotypic studies have shown that most thymocytes with a C-value of 2.0 have in fact left the cell cycle and are thus not G1 cells but rather G0 cells (2). Noncycling G0 cells comprise 70–80% of the total adult thymocytes and are primarily small TCR^{lo} $CD4^+8^+$ cells that die intrathymically without further proliferation (nor can they be experimentally induced to proliferate) (31). In contrast, the remaining G0 cells, comprising 10–15% of total adult thymocytes are mature TCR^{hi} $CD4^+8^-$ cells. These cells can be stimulated to reenter the cell cycle after cross-linking of the TCR. To distinguish between G0 and G1 cells in each fraction, the percentages of small TCR^{hi} $CD4^+8^+$ and TCR^{hi} thymocytes were obtained by three color flow cytometry. As shown in Table I, both types of G0 cells were

Table I. Cell Cycle Analysis of Elutriated Mouse Thymocytes

Fraction	Flow	Vol*	G0/G1‡	S_E‡	S_L‡	G2/M‡	"C" value§	TCR Hi‖	Small $CD4^+8^+$‖	G0¶
	ml/min	*μm^3*	%	%	%	%		%	%	%
Unfractionated			73	10	7	10		13	40	53
2 $G0_A$	12	39	98	–	–	–	2.0	10	85	95
3 $G0_A$	15	48	85	15	–	–	2.0	28	61	89
4 $G0_B$	16	87	90	10	–	–	2.0	27	34	61
5 $G1/S_1$	18	97	82	15	3	–	2.0	15	21	36
6 $G1/S_2$	20	106	60	20	12	8	2.3	9	13	22
7 $G1/S_3$	22	135	45	20	14	21	2.6	6	10	16
8 S	24	165	30	25	22	23	2.8	6	6	12
9 S/G2	26	184	12	22	24	42	3.0	6	4	10
10 G2/M	28	223	8	14	28	50	3.4	5	2	7

* Volume calculated using a Coulter Channalyzer.
‡ Cell cycle percentages calculated using the INTGRA program (Coulter Electronics).
§ "C" value calculated using the mean channel position obtained in the DNA histograms presented in Fig. 1.
‖ Percentages determined by staining with anti-TCR, CD4 and CD8 antibodies and analyzing by flow cytometry (reference 16).
¶ The total percentage of G0 cells determined by adding the percentages of TCR and $CD4^+8^+$ cells together.

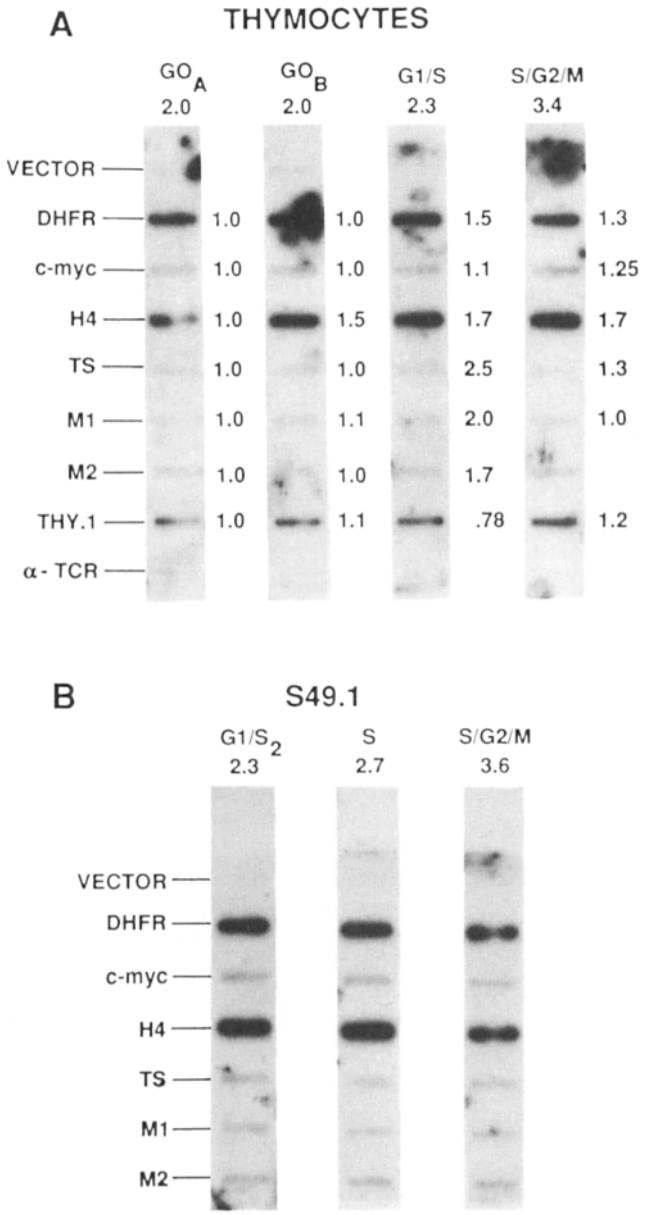

Figure 2. (*A*) Nuclear run-on transcription analysis of the 5′ end of the DHFR gene, c-myc, histone H4, TS, M1, M2, THY-1, and α-TCR genes in elutriated neonatal mouse thymocytes. An equal number of cpms was hybridized to filters containing double-stranded probes for each of the genes. Hybridization (over background to the vector) is detectable for all genes with the exception of the α-TCR gene. The cell cycle position and average DNA content for each fraction are listed above the filter and densitometric values are presented next to each filter. The hybridization obtained with $G0_A$ was set as 1.0 and the signals obtained through the cell cycle were compared to that. (*B*) Nuclear run-on transcription analysis of the same genes in elutriated mouse T-cell line S49.1. Although the intensities of hybridization are greater due to the greater input of labeled RNA, the relative hybridization for each gene at each cell cycle position is essentially identical.

found predominantly in the first four fractions. These data combine to indicate that the thymus at this stage of maturation contains cycling and noncycling cells that can be separated by centrifugal elutriation.

A Comparison of Transcription Rates of Various Genes in Elutriated Mouse Thymocytes and in a T-Cell Line, S49.1

To compare the rates of transcription of various growth-related genes, run-on transcription assays were performed on nuclei isolated from either thymocytes or T-cell line S49.1 after preparation of cell cycle–specific stages by centrifugal elutriation (Fig. 2). Labeled nascent transcripts were hybridized to slot blots containing an excess of double-stranded DNA for each of the genes. The resulting autoradiogram was quantitated by densitometry, and values are shown on the figure. All of the hybridizing signals were eliminated when the run-on reactions were done in the presence of 1 μg/ml α-amanitin, indicating that the hybridizing labeled RNAs were generated by RNA polymerase II (data not shown). The high signal within the 5′ end of the DHFR gene when compared to the other "housekeeping genes" reflects a disproportionate amount of synthesis at the 5′ end of the gene and not the rate of transcription for the entire gene (data not shown). A detailed analysis of run-on transcription across the DHFR gene will be presented elsewhere. Thymocytes were observed to have low but detectable levels of transcription for all genes studied except α-TCR (note that small amounts of vector hybridization were observed in the thymocyte samples on some filters). Whereas the RNA from the $G0_B$ cells showed faint hybridization to the vector, the RNA from the G1/S cells did not. Based on repetitive experiments where no hybridization to the vector was observed, we conclude that the signal produced from the TS, M1, and M2 sequences, albeit weak, is not spurious hybridization. In fact, this signal represents the rates of transcription at these genes. Surprisingly, the G0 fractions produced nearly the same amount of nascent DHFR, *c-myc*, M1, M2, histone H4, and THY-1 RNAs as the proliferating cells. By comparing the amount of hybridization obtained in each fraction to that obtained in the $G0_A$ fraction, we observed a small but reproducible increase in the transcription rate for the TS gene in cells that bordered between proliferating and nonproliferating states. Elutriated S49.1 cells, which represent cells in a physiological continuous cell cycle, gave similar results. These results show, at least qualitatively, that the rates of transcription for these genes are similar in freshly isolated proliferating thymocytes and in a transformed T-cell line.

The fact that double-stranded probes were used on the slot blots necessitates a qualified explanation of these data. Although it is highly unlikely (given the number of probes used), it is possible that a balancing of sense and antisense transcription could give the impression of an absence of transcriptional regulation. Antisense transcription is known to exist in the 5′ end of the DHFR gene (9) and throughout the *c-myc* gene (26). However, the DHFR cDNA sequence used on the slot is sufficiently 3′ of the opposite strand transcription unit, so that its RNA is not detectable. The *c-myc* double-stranded second exon sequence has been previously shown to be capable of detecting changes in the *c-myc* transcription rate (14). Therefore, the signals detected with these sequences most probably reflect the transcription rates at these loci. For the remaining genes, little is known about the rates of transcription across their genomic loci, therefore, we cannot rule out the possibility that antisense transcription may affect our interpretations.

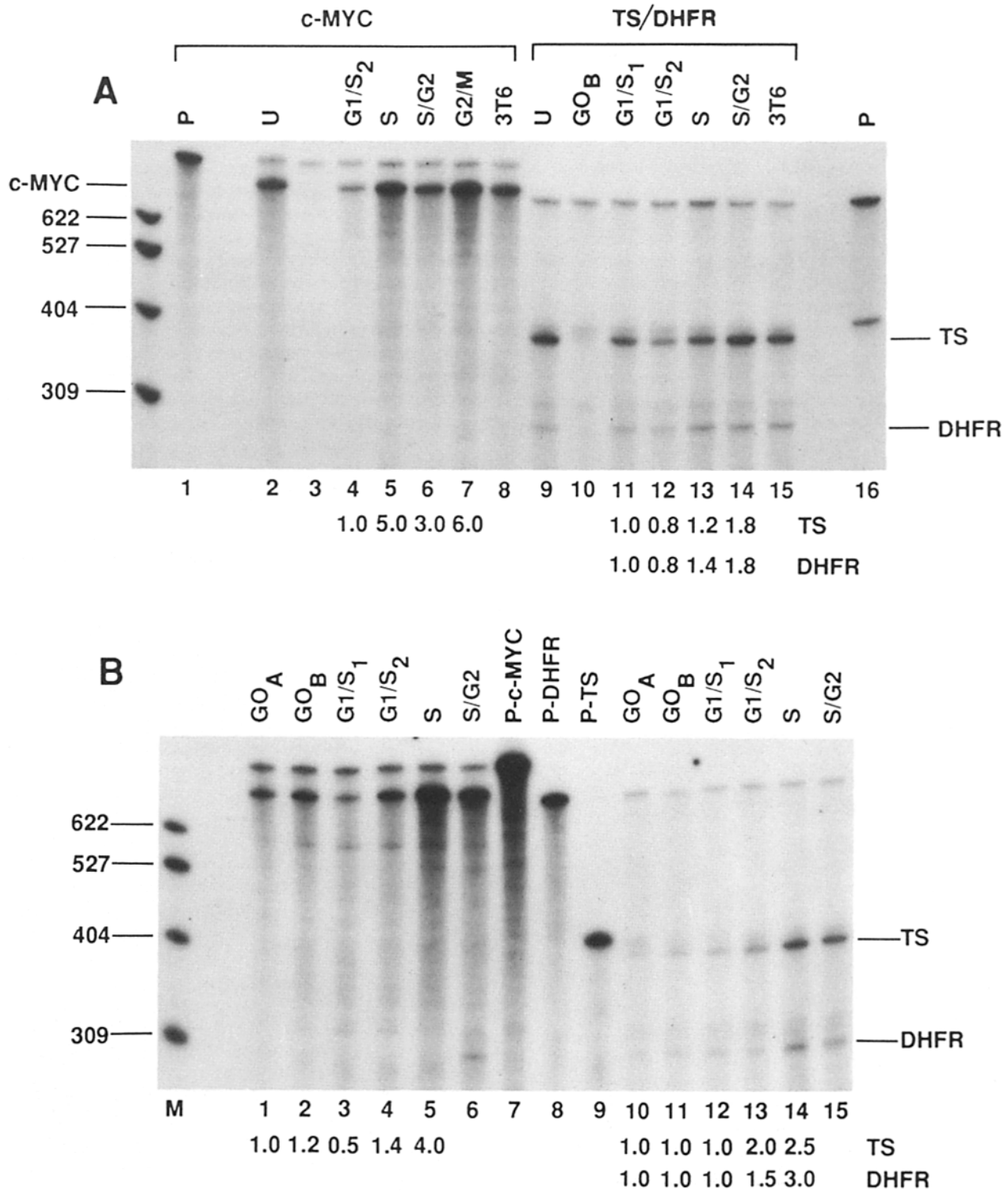

Figure 3. (*A*) Autoradiogram of an RNase protection experiment on thymocyte-elutriated RNA. Equal amounts of RNA (2 μg) were annealed to 2 × 10^6 cpm of probe and then digested with RNase. (lane *M*) pBR322/Msp I [^{32}P-dCTP]-labeled restriction fragments; (lane *1*) *c-myc* probe alone; (lane *2*) *c-myc*–protected products in RNA from unfractionated cells; lane *3* contains no RNA; (lanes *4–7*) *c-myc*–protected products in RNA isolated from elutriated cell fractions; (lane *8*) *c-myc*–protected products in RNA isolated from a cultured cell line 3T6; (lane *9*) DHFR- and TS-protected products in RNA from unfractionated cells; (lanes *10–14*) DHFR- and TS-protected products in RNA isolated from elutriated cell fractions; and (lane *15*) DHFR- and TS-protected products in RNA isolated from 3T6 cells. The full-length protected fragments for each gene are indicated at the side of the autoradiogram. The densitometric values are presented under the autoradiogram. (*B*) Autoradiogram of an RNase protection experiment carried out on thymocyte RNA obtained from a separate elutriation that included samples from the G0 cells. (lanes *1–6*) c-myc–protected products in RNA isolated from elutriated cell fractions; (lanes *7–9*) *c-myc*, DHFR, and TS probes, respectively; and (lanes *10–15*) DHFR- and TS-protected products in RNA isolated from elutriated cell fractions. The densitometric values are presented under the autoradiogram.

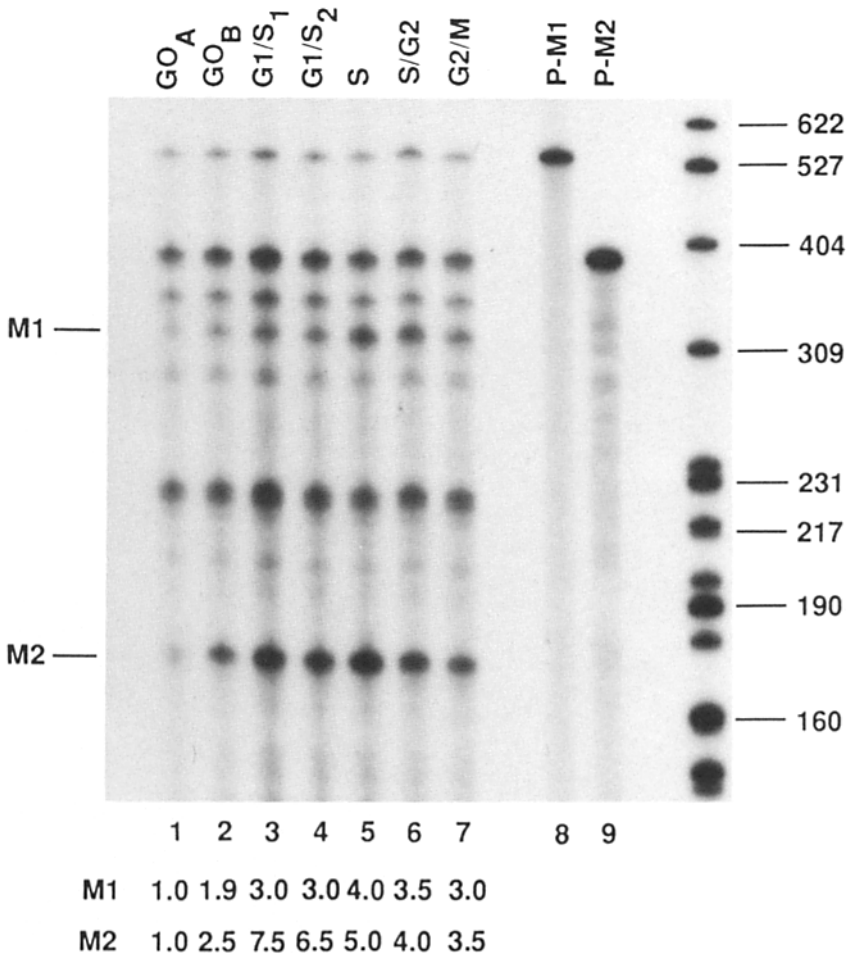

Figure 4. Autoradiogram of an RNase protection experiment on thymocyte RNA prepared after elutriation using probes for the ribonucleotide reductase subunits M1 and M2. (lanes *1–7*) M1- and M2-protected products in RNA isolated from elutriated cell fractions; lanes *8* and *9* contain the probes alone. The lines on the side of the autoradiogram indicate the positions of the full-length protected products; the other bands on the gel, other than the undigested probes are nonspecific and obtainable using tRNA as carrier (data not shown). The densitometric values are presented under the autoradiogram for each subunit.

Analysis of Steady-State mRNA Levels

Analysis of transcription rates suggested that all of the growth-related genes studied with the exception of the TS gene were transcribed in the thymocytes irrespective of growth status or cell cycle position. To investigate this more completely, total RNA was isolated from the various elutriated thymocyte fractions, and equal amounts were analyzed in RNase protection assays to measure the steady-state mRNA levels. In Fig. 3 an analysis of DHFR and TS expression is presented, based on results from two separate experiments. Both RNAs are barely detectable in the G0 fractions but showed gradual accumulation in the proliferating cells with maximum levels found in the last (G2) fraction (Fig. 3 *A*, lanes *10–14*). A comparable level of these RNAs was found in cultured mouse 3T6 cells (Fig. 3 *A*, lane *15*). In a second experiment, the fraction most enriched in G0 cells, $G0_A$, was included (Fig. 3 *B*, lane *10*). Again, very little DHFR or TS mRNA was observed in these cells (Fig. 3 *B*, lanes *10–12*), but the steady-state levels increased in the G1/S cells. The calculated numerical increase appears dramatic because the comparison is between RNA levels in the GO and S phase cells, in contrast to the results in Fig. 3 *A*, in which G1/S and S phase cells were used as the standard. These data indicate that the accumulation of both the DHFR and TS mRNAs is closely dependent on the proliferating status of the cell.

Surprisingly, *c-myc* expression was different. A reproducible three- to fivefold increase in *c-myc* steady-state RNA levels was observed in cells progressing from G1/S to S phase (Fig. 3 *A* and *B*, lanes *4* and *5*). The level remained elevated in the later stages of the cycle (Fig. 3 *A*, lane *7*). The small decrease in the S/G2 cells (Fig. 3, *A* and *B*, lane *6*) and increase in the G2/M cells (Fig. 3 *A*, lane *7*) was also reproducible in repeated experiments. A similar biphasic pattern of expression for the *c-myc* gene has been previously noted (30). In contrast to the pattern observed with DHFR and TS, appreciable amounts of *c-myc* RNA were found in fractions comprised of 60–90% G0 cells (Fig. 3 *B*, lanes *1* and *2*).

The patterns found with the two subunits of ribonucleotide reductase are qualitatively similar and parallel to *c-myc* rather than to DHFR and TS. The M1 subunit RNA was found to be present in low but detectable levels in the G0 cells (Fig. 4, lane *1*), followed by a threefold increase in the dividing cells (Fig. 4, lanes *3–7*). M2 subunit RNA was also low in the G0 fractions (Fig. 4, lanes *1* and *2*), but showed an abrupt 7.5-fold increase as the percentage of G0 cells decreased and the amount of G1/S-phase cells increased (Fig. 4, lanes *2* and *3*). An appreciable decrease was also observed in the later stages of the cell cycle, whereas the level of M1 RNA remained relatively constant (Fig. 4, lanes *4–7*).

For the purpose of comparison, we also quantitated steady-state message levels for a gene that has been proven to show cyclic regulation, the mouse histone H4 gene (6). There was no detectable RNA in the G0 cells but a peak of accumulation in the S-phase fraction of approximately

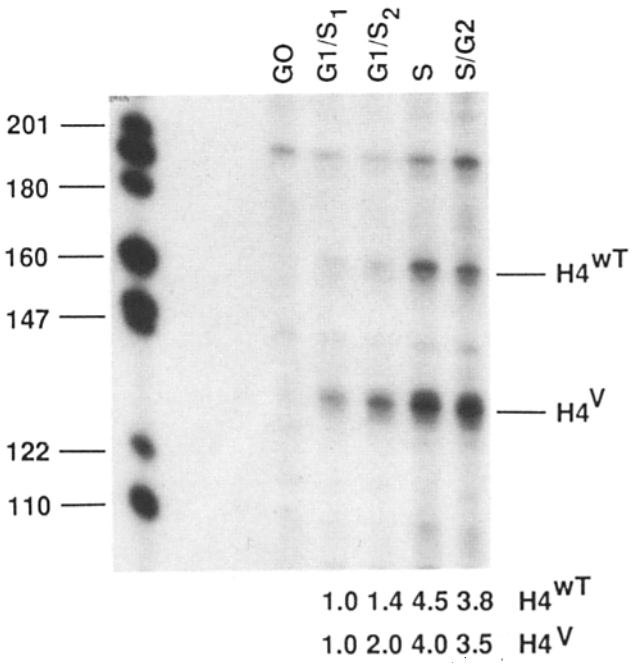

Figure 5. Autoradiogram of an RNase protection experiment on thymocyte RNA prepared after elutriation using a probe for the histone H4 gene. (lanes *1–5*) H4-protected products in RNA isolated from elutriated cell fractions. The lines on the side of the gel indicated the position of the full-length protected products from the wild-type (*WT*) and variant (*V*) H4 RNAs, the remaining bands are nonspecific and obtainable with tRNA (data not shown). Densitometric values are presented below the autoradiogram.

fivefold over that found in the G1/S cells, was followed by a slight decrease in the S/G2/M enriched cells (Fig. 5). Taken together, these data indicate that these cell proliferation–associated genes exhibit different patterns of expression and accumulation through the cell cycle and that these differentials are most likely the result of posttranscriptional regulation.

Discussion

The analysis of the mammalian cell cycle has depended heavily on the use of cultured cells as models for how genes are regulated during the process of "normal" proliferation. The nature of this dependence is both historical and technical. Recent advances in the field of counter-flow centrifugation now make it possible to separate cells into cell cycle stage-specific populations without artificially inducing cell synchrony by either serum starvation or drug inhibitions. The only requirements are that the cells be actively dividing, easily dissociable into single cell suspensions, and have relatively uniform volume changes associated with cell cycle progression. One organ that satisfies these requirements is the thymus. In this study we have used elutriated mouse thymocytes to extend our previous studies on the regulation of the DHFR gene in cultured cells to include rate of transcription studies and to investigate the regulation of other genes associated with cell proliferation. Thymuses from neonatal mice contain a mixture of cells that differ in maturational stage and proliferative potential, and using these cells we have been able to assess the changes in gene expression associated not only with cell cycle progression but also in cells that have undergone a developmental decision to withdraw from the cycle. With this system we have asked whether the results obtained with cultured cells truly reflect what is found in an organism. We approached this question with the expectation that the genes under analysis would fall into one of three classes: (*a*) genes that are expressed regardless of the growth status of the cell or cell cycle position; (*b*) genes that are invariably expressed only in actively dividing cells; and (*c*) genes that show periodic synthesis or degradation during a particular stage of the cell cycle. The DHFR gene clearly falls into the second category. The DHFR transcription rate is constant regardless of growth status or cell cycle position, yet appreciable levels of mRNAs are found only in the proliferating cells. No dramatic cell cycle–dependent increase or decrease in steady-state levels is observed. The results with DHFR confirm our cell culture observations (11) and strengthen the conclusion that the DHFR gene is only growth regulated, i.e., the increase in steady-state level merely reflects growth-dependent increases in total cellular RNA. The TS gene may belong to the third class, showing a transient increase in the rate of transcription which temporally correlates with the observed increases in steady-state levels. Previous studies have shown that the TS gene is expressed only in proliferating cells (22) and that this expression is invariant through the cell cycle (17). The molecular mechanisms responsible for the proliferation-associated increase have been claimed to be both transcriptional (22) and posttranscriptional (7). Our results suggest that TS mRNA persists in the absence of elevated transcription rate, suggesting additional posttranscriptional events are involved. Therefore, both forms of regulation are utilized in the regulation of the TS gene in developing T lymphocytes.

The *c-myc* gene delineates a different situation. This gene has a complex mode of regulation and is commonly thought to be expressed only in proliferating cells (21, 29). Previous work carried out on a variety of cultured cells types as well as on elutriated chicken thymocytes has shown that *c-myc* RNA levels are invariant through the cell cycle (36). Our results demonstrate that there is an appreciable level of *c-myc* RNA in thymocytes which have withdrawn from the cell cycle, and that there is a fivefold increase in steady-state mRNA as these cells proceed through G1 into S. The difference in frequency of G0 cells between these fractions was <10%, therefore, it is unlikely that the increase in RNA results from a greater number of proliferating cell types in one fraction versus another. During the transition from G1 to S, the volume of these cells increases approximately twofold (Table I), therefore, the fivefold increase in *c-myc* mRNA steady-state level represents a real increase in the concentration of mRNA molecules.

In cultured cells, the tight correlation between the presence of the *c-myc* protein and the proliferation of the cell, has led to the conclusion that *c-myc* has an important function in controlling cell division (17, 29). Recent results have implied that the putative helix-loop-helix DNA binding domain of the *myc* family of genes is an important component of proteins that have primary roles in establishing cell identity, e.g., *daughterless* and *Myo D1* (24, 34). This suggests that the *c-myc* protein itself may have additional developmental functions. The differential expression of two *c-myc* pro-

tooncogenes during *Xenopus* development also supports this postulate (38). Since we observed *c-myc* mRNA in both proliferating and nonproliferating thymocytes, the large increase observed during the G1/S to S transition may represent synthesis in response to a developmental demand for additional product and not for functions associated with proliferation. The differences between these results and those previously reported (36) could result from the different species, ages, and types of the thymocytes used. We have used 7-d-old mice, whereas Thompson et al. (36) used 6-wk-old chickens. If the increase we observed at the G1/S boundary is developmentally controlled, it might not be present in thymocytes derived from a later stage animal.

As observed with DHFR, the *c-myc* transcription rate was relatively constant throughout the cell cycle as well as in the G0 cells. This result agrees with observations in at least one cultured cell line in which the rate of *c-myc* transcription was unaffected when a G0 state was induced by serum starvation (1). The DNA construct we used to measure transcription rate contains only the second exon, therefore, the signal obtained on the slot-blot was representative of events further 3′ of the intragenic pausing that takes place in the first exon of the *c-myc* gene (26). Since the intensity of the run-on signal did not increase in relation to the increase in *c-myc* mRNA steady-state levels, the molecular mechanism for this increase must involve a posttranscriptional process and not be the result of release of intragenic pausing. Overall, the *c-myc* gene falls into two classes, a gene that is expressed at low levels in thymocytes, proliferating or not, and a gene that is upregulated at the G1/S boundary. If this upregulation is developmentally controlled and not cell cycle controlled, it would be an aspect of genetic regulation that might not be observed in a cultured cell.

The ribonucleotide reductase subunits M1 and M2 showed slightly different patterns of regulation; the difference reflects the variation observed between the M1 and M2 proteins (8). The activity of the holoenzyme is modulated by the level of the catalytic subunit M2, whose activity increases three fold during S phase, whereas the activity of the M1 subunit remains constant. Our results showed that the levels of steady-state RNA for these two genes parallel the protein changes. Whereas the M1 mRNA is found in all proliferating cell fractions in approximately equal amounts, the M2 mRNA reaches a higher steady-state level at the beginning of S phase. Recent results using L1210 cells separated into cell cycle stage-specific populations by centrifugal elutriation, have shown that the M2 subunit RNA reaches a higher steady-state level in S phase cells than does the M1 subunit RNA, and then decreases as the cell progresses to G2/M (37). These data from cell cultures are in general agreement with those presented here. Again, the transcription rates of M1 and M2 are invariant through the cell cycle. Finally, a comparison of thymocytes and a cultured T-cell line, S49.1, suggests that at least one process of cell immortalization has not affected the pattern of regulation for these "housekeeping" enzymes.

Specific transcriptional control is a common theme in the study of differentiation and cell type-specific gene expression. Our results with genes common to virtually all cell types suggest, however, that regulation at the level of initiation of transcription is a minor component of processes that modulate transcript levels, at least in thymocytes. It should be emphasized that this conclusion is based on the assumption that during preparation of nuclei, regulatory interactions are not disturbed such that the nuclear run-on technique accurately assesses the in vivo transcription rate of a particular gene.

In summary, neonatal mouse thymocytes provide a valuable model for cell cycle expression studies that include measurements of rates of transcription and steady-state mRNA levels. Analyses of gene expression in proliferating and nonproliferating thymocytes of different maturational stages can be made simultaneously, and the contribution of developmental events to the pattern of cell cycle-dependent expression can be evaluated. Finally, analysis of gene expression in these cells has shown that the pattern of expression for the dihydrofolate reductase gene is the same in vivo as it is in cultured cells, as are the rates of transcription across the cell cycle for other genes involved in the biosynthesis of nucleotide precursors.

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References

1. Blanchard, J., M. Piechaczyk, C. Dani, J. Chambard, A. Franchi, J. Pouyssegur, and P. Jeanteur. 1985. *c-myc* gene is transcribed at a high rate in G_0-arrested fibroblasts and in post-transcriptionally regulated in response to growth factors. *Nature (Lond.).* 317:443–445.
2. Boyer, P. D., R. A. Diamond, and E. V. Rothenberg. 1989. Changes in inducibility of IL-2 receptor α-chain and T cell receptor expression during thymocyte differentiation in the mouse. *J. Immunol.* 142:4121–4130.
3. Chang, A. C. Y., J. H. Nunberg, R. J. Kaufman, H. A. Erlich, R. T. Schimke, and S. N. Cohen. 1978. Phenotypic expression in *E. coli* of a DNA sequence for mouse dihydrofolate reductase. *Nature (Lond.).* 275:617–624.
4. Chen, Y., D. Becker, T. Lindsten, M. Okamura, D. Cohen, and M. Davis. 1984. A third type of murine T-cell receptor gene. *Nature (Lond.).* 312:31–35.
5. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
6. Chrysogelos, S., D. E. Riley, G. Stein, and J. Stein. 1985. A human histone H4 gene exhibits cell cycle-dependent changes in chromatin structure that correlate with its expression. *Proc. Natl. Acad. Sci. USA.* 82:7535–7539.
7. Chung-Her, J., P. K. Geyer, and L. F. Johnson. 1985. Control of thymidylate synthase mRNA content and gene transcription in an overproducing mouse cell line. *Mol. Cell. Biol.* 5:2527–2532.
8. Eriksson, S., and D. W. Martin. 1981. Ribonucleotide reductase in cultured mouse lymphoma cells. *J. Biol. Chem.* 256:9536–9440.
9. Farnham, P. J., J. M. Abrams, and R. T. Schimke. 1985. Opposite-strand RNAs from the 5′-flanking region of the mouse dihydrofolate reductase gene. *Proc. Natl. Acad. Sci. USA.* 82:3978–3982.
10. Feder, J., L. Yen, E. Wijsman, L. Wang, L. Wilkins, J. Schroder, N. Spurr, H. Cann, M. Blumenberg, and L. L. Cavalli-Sforza. 1985. A systematic approach for detecting high-frequency restriction fragment length polymorphisms using large genomic probes. *Am. J. Hum. Genet.* 37:635–649.
11. Feder, J. N., Y. G. Assaraf, L. C. Seamer, and R. T. Schimke. 1989. The pattern of dihydrofolate reductase expression through the cell cycle in rodent and human cultured cells. *J. Biol. Chem.* 264:20583–20590.
12. Fowlkes, B. J., and D. M. Pardoll. 1989. Molecular and cellular events of T cell development. *Adv. Immunol.* 44:207–264.
13. Geyer, P. K., and L. F. Johnson. 1984. Molecular cloning of DNA sequences complementary to mouse thymidylate synthase mRNA. *J. Biol. Chem.* 259:7206–7211.
14. Greenberg, M. E., and B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature (Lond.).* 311:433–483.

15. Grosschedl, R., and D. Baltimore. 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. *Cell.* 41:885–897.
16. Guidos, C. J., I. L. Weisseman, and B. Adkins. 1989. Intrathymic maturation of murine T lymphocytes from CD8+ precursors. *Proc. Natl. Acad. Sci. USA.* 86:7542–7546.
17. Hann, S. R., C. B. Thompson, and R. H. Eisenman. 1985. *c-myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature (Lond.).* 314:366–369.
18. Hiraki, D. D., D. Nomura, T. Yokota, K. Arai, and R. L. Coffman. 1983. Isolation and characterization of expressible cDNA clones for mouse Thy-1: a model system for cDNA expression of cell surface proteins. *J. Immunol.* 136:4291–4296.
19. Horibata, K., and A. W. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell Res.* 60:61–77.
20. Iman, A. M. A., P. H. Crossley, A. L. Jackman, and P. F. R. Little. 1987. Analysis of thymidylate synthase gene amplification and of mRNA levels in the cell cycle. *J. Biol. Chem.* 262:7368–7373.
21. Kelly, E., B. H. Cochran, C. D. Stiles, and P. Leder. 1983. Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell.* 35:603–610.
22. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
23. Murrary, A. W., and M. W. Kirschner. 1989. Dominoes and clocks: the union of two views of the cell cycle. *Science (Wash. DC).* 246:614–621.
24. Murre, C., P. S. McCaw, and D. Baltimore. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell.* 56:777–783.
25. Navalgund, L. G., C. Rossana, A. J. Muench, and L. F. Johnson. 1980. Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. *J. Biol. Chem.* 255:7386–7390.
26. Nepveu, A., and K. B. Marcu. 1986. Intragenic pausing and anti-sense transription within the murine *c-myc* locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2859–2865.
27. Nepveu, A., R. A. Levine, J. Campisi, M. E. Greenberg, E. B. Ziff, and K. B. Marcu. 1987. Alternative modes of c-*myc* regulation in growth factor-stimulated and differentiating cells. *Oncogene.* 1:243–250.
28. Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay. 1989. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA.* 86:1614–1618.
29. Rabbitts, P. H., J. V. Watson, A. Lamond, A. Forster, M. A. Stinsom, G. Evan, W. Fischer, E. Atherton, R. Sheppard, and T. H. Rabbitts. 1985. Metabolism of *c-myc* gene products: *c-myc* mRNA and protein expression in the cell cycle. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2009–2015.
30. Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover. 1986. Sequential expression of proto-oncogenes during lectin-stimulated mitogenesis of normal lymphocytes. *Proc. Natl. Acad. Sci. USA.* 83:3982–3986.
31. Rothenberg, E. V., and J. P. Lugo. 1985. Differentiation and cell division in the mammalian thymus. *Dev. Biol.* 112:1–17.
32. Scollay, R., J. Smith, and V. Stauffer. 1986. Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol. Rev.* 91:129–157.
33. Sheng-ong, G. L. C., J. Keath, S. P. Piccoli, and M. D. Cole. 1982. Novel *myc* oncogene RNA from abortive immunoglobulin-gene recombination in mouse plasmacytomas. *Cell.* 31:443–452.
34. Tapscott, S. J., R. L. Davis, M. J. Thayer, P. Cheng, H. Weintraub, and A. B. Lasser. 1988. MyoD1: A nuclear phosphoprotein requiring a *myc* homology region to convert fibroblast to myoblasts. *Science (Wash. DC).* 241:405–411.
35. Thelander, L., and P. Berg. 1986. Isolation and characterization of expressible cDNA clones encoding the M1 and M2 subunits of mouse ribonucleotide reductase. *Mol. Cell Biol.* 6:3433–3442.
36. Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine. 1985. Levels of *c-myc* oncogene mRNA are invariant throughout the cell cycle. *Nature (Lond.).* 314:363–366.
37. Thompson, D. P., G. L. Carter, and J. G. Cory. 1989. Changes in messenger RNA levels for the subunits of ribonucleotide reductase during the cell cycle of leukemia L1210 cells. *Cancer Comm.* 1:253–260.
38. Vriz, S., M. Taylor, and M. Michali. 1989. Differential expression of two *Xenopus c-myc* proto-oncogenes during development. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4091–4097.
39. Zinn, K., D. DiMaio, and T. Maniatis. 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. *Cell.* 34:865–879.