
Post-transcriptional regulation of the chicken thymidine kinase gene

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ABSTRACT

In attempting to understand the molecular basis of the control of chicken thymidine kinase (cTK) gene expression, we have examined the steady state cTK RNA content, and the patterns of DNA methylation, chromatin structure and endogenous nuclear runoff transcription of this gene in dividing and non-dividing cells. Our results reveal that the steady state level of cTK poly A⁺ RNA is correlated with the divisional activity of normal avian cells and tissues. However, no differences in the pattern of Hpa II site methylation or chromatin structure are found among cells containing high or undetectable levels of steady state cTK RNA. In addition, no differences in cTK transcription as assayed by nuclear runoff experiments are detectable in isolated nuclei derived from dividing or non-dividing cells containing high or low levels of steady state cTK RNA. These results suggest that the principal control of chicken thymidine kinase gene expression is post-transcriptional in nature.

INTRODUCTION

During development, specific genes become activated in different cell lineages at specific times. For example, at approximately 32 hours of incubation, hemoglobin is first detectable in erythroblasts of the developing chicken embryo, whereas neither globin peptides nor globin transcripts are detectable in precursor erythroid cells at 20 hours.^{1,2} The differential expression of such tissue specific genes appears to be correlated with changes in chromatin structure. In the case of the chicken α -globin genes in committed erythroid cells there is a very tight correlation between the expressed gene, the gene that is transcribed, the gene that is undermethylated and the gene that is very sensitive to DNase I and which contains characteristic nuclease hypersensitive sites at its 5' side.³ Non-erythroid cells display none of these chromatin changes in the α -globin locus. In addition, examination of the developmental activation of globin genes in the hematopoietic lineage has revealed that such changes in chromatin structure are not present in progenitor erythroid stem cells, but these changes appear

to coincide with the developmental activation of any one particular globin gene.^{2,3}

While these sorts of correlations have been made for a number of tissue specific genes (e.g., globin¹⁻³, ovalbumin^{4,5}, vitellogenin^{6,7}), very little information is available concerning the molecular correlates or controls of genes that are expressed in a non tissue-specific fashion. One of the most extensively studied of these ubiquitous activities is the enzyme thymidine kinase (TK) which phosphorylates thymidine and salvages the nucleoside for DNA synthesis. Several laboratories have demonstrated that TK activity is maximal during the log phase of growth and diminishes as cells grow to confluency in a number of different cell culture systems.⁸⁻¹¹ In most of these systems, TK activity is barely detectable in G1, increases and peaks in S phase and decreases as cells progress through G2 to mitosis.¹²⁻¹⁷

Interestingly, the cell-cycle regulation of TK activity has also been observed upon transfection of cellular, but not viral TK genes into mouse cell lines¹⁸, indicating that the molecular determinants of cellular TK gene activity are closely linked to or determined by the coding sequence of this gene. Although the molecular basis of the cell cycle regulation of the endogenous or transfected cellular TK genes is not known, it has been reported that in the case of an observed high frequency of switching in expression of the herpes simplex virus (HSV) TK gene transfected into mouse fibroblasts, this switching in activity was correlated with changes in chromatin structure.¹⁹

In attempting to understand the molecular basis of the regulation of expression of cellular TK gene activity, we have investigated the steady state RNA levels, DNA methylation, chromatin structure and nuclear transcription of the chicken TK gene in chicken cell lines, cultures and tissues in various states of mitotic activity. Our results indicate that while large differences in the steady state levels of TK mRNA are apparent in cells with different mitotic activity, no differences in the chromatin structure or transcription of the TK gene is detectable among such cells. Thus, the chicken TK gene appears to be regulated at the post-transcriptional level.

MATERIALS AND METHODS

Cells

Chick embryo fibroblasts (CEF) were dissected from the region of the developing breast muscle of 11 day old virus-free chicken embryos and grown

as described previously.¹ Cultures of myoblasts and myotubes were also derived from the same region of the embryo, but grown as described by Kligman & Nameroff.²⁰ Replicating myoblasts were eliminated from the myotube cultures by treatment with cytosine arabinoside as described by Yeoh & Holtzer.²¹ Red blood cells (rbc) were obtained by vein puncture from an 8 week old chicken, which was made anemic with phenylhydrazine.¹ Brain and bursa were dissected from a 4 week old chicken, and bursal lymphocytes were separated from the stroma of this organ by scraping with a scalpel. MSB1 cells, a chicken T-cell line transformed by Marek's virus^{22,23} were maintained in RPMI 1640 medium supplemented with 1% chicken serum (Gibco) and 5% calf serum (Gibco). The various RP-9 cell populations are chicken B-cell lines transformed by the RAV-1 virus and were grown as described.²⁴

Preparation of RNA

Total cellular RNA was prepared from cells and tissues using a guanidinium isothiocyanate procedure, adapted from Ullrich *et al.*²⁵ Tissue or cell pellets were resuspended in buffer containing 5 M guanidinium isothiocyanate, 5 mM sodium citrate, (pH 7.0), 0.7 M β -mercaptoethanol and 50 mM EDTA, at a ratio of about 10 volumes buffer:1 volume cells (or tissue). The guanidinium solution was filtered, treated with diethyl pyrocarbonate (DEP) and autoclaved before use. After vigorous homogenization, using a motor driven homogenizer with a teflon pestle, the cellular extract was loaded onto 1.5 ml of cesium chloride (5.7 M), EDTA (50 mM) and centrifuged in a Beckman SW50.1 rotor for 16 hrs at 20°C. Following pelleting of the RNA, the supernatant was carefully removed using a pasteur pipette, gently decanting the last 1 ml of solution. The inside of the tube was then wiped with a sterile cotton tipped applicator and the pellet resuspended in DEP (diethyl pyrocarbonate) treated 0.1 M sodium acetate containing 0.5% SDS. The resuspended RNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1), before ethanol precipitation with 2.5 volumes of ethanol overnight at -20°C. The RNA precipitate was pelleted in a Sorval RC5B (10,000 rpm, 20 minutes) and the pellet washed with 70% ethanol, dried under vacuum and resuspended in (DEP treated) distilled water.

Polyadenylated RNA was isolated from the total RNA using oligo-dT-cellulose as described by Aviv and Leder.²⁶

Hybridization of RNA

Preparation of RNA "dot blots" was performed essentially as described by White and Bancroft.²⁷ Briefly, the poly-A⁺ RNA was suspended in water and

mixed with an equal volume of 20 X SSC (1 X SSC equals 0.15M NaCl, 0.015 M Sodium Citrate), 37% formaldehyde (3:2). The mixture was then heated to 65°C for 10 minutes and cooled on ice. Sufficient 20 X SSC was then added to bring the final salt concentration to 15 X SSC. The RNA was diluted to the required concentration in 15 X SSC and applied to presoaked nitrocellulose, equilibrated with 15 X SSC and mounted in a Schleicher & Schuell "minifold" apparatus using S & S 470 paper as backing. After binding of the RNA the filter was dried under a heat lamp and baked under vacuum for 2 hours at 80°C.

Electrophoresis and transfer of RNA to nitrocellulose was performed using the formaldehyde gel system of Seed and Goldberg.²⁸ Samples were denatured for 60 minutes at 65°C in 50% formamide, 6.5% formaldehyde and 1 X MOPS buffer, loaded on agarose gels containing 2.2 M formaldehyde and run in 1 X MOPS buffer (0.2 M Morpholinopropane sulphanic acid pH 7.0, 50 mM sodium acetate, 1 mM EDTA). Transfer to nitrocellulose²⁹, was performed using 20 X SSC as transfer buffer. Transfer was allowed to continue for 16 hrs, after which the blot was dried under a heat lamp and baked as above.

Hybridization was under conditions described previously³⁰ using "Starks" buffer³¹ for prehybridization and the same buffer supplemented with 10% dextran sulphate and approximately 5×10^6 cpm of ³²P-labelled probe (see below). Hybridization was overnight at 42°C, after which the blot was washed twice in 2 X SSC, 0.1% SDS at room temperature and twice at 55°C in 0.1 X SSC, 0.1% SDS, air dried and exposed at -80°C using Kodak XAR-5 film with Dupont Cronex (brand) enhancing screens. Exposure times varied from 16-48 hours.

The RNA blots were hybridized to nick-translated double-stranded DNA probes consisting either of a 3.2 kbp Sst I fragment containing cellular myc (c-myc) sequences described previously³², or a 2.9 kbp Hind III fragment containing chicken thymidine kinase (TK) sequences.³³ Preparation of plasmid DNA, isolation of fragments and nick-translation, were performed as described.³⁴ The cTK plasmid was a gift from M. Wigler.

Preparation and hybridization of DNA

Nuclei from cells and tissue were isolated, DNA prepared and blot-hybridized, and DNase I digestions of isolated nuclei were performed as described.³⁵ Restriction enzyme digestions were performed according to manufacturer's specifications.

Nuclear transcription and isolation and analysis of RNA

Transcription of nuclei and subsequent isolation of ³²P-labelled RNA were

performed as described previously³⁶, except that the reaction volumes were increased to 750 ul and the number of nuclei was increased to that containing 500 ug of DNA. In addition, rather than using double-stranded DNA fragments immobilized on nitrocellulose for analysis of the specific sequence content of the run off products, single stranded DNA molecules were used. Single stranded cloned DNA's were prepared in the single-stranded DNA vectors M13mp8, mp9, mpl0, or mpl1 as described.³⁷ The c-myc sequences cloned into these vectors correspond to both strands of the 3.2 kbp myc region described above, or approximately 1.7 kbp consisting of the intron and second exon of this gene.²⁴ The c-myc single stranded cloned DNA's were gifts of W. Schubach. The cTK single stranded DNA's, gifts of S. McKnight, consisted of approximately 1.5 kb, corresponding to both strands of the region of cellular gene between the EcoRI and Bgl II sites depicted in Figure 2B. The use of these single stranded DNA's permitted detection of c-myc and cTK sequences within the run off transcription products, whereas such sequences were below the limit of detection when the ³²P-nuclear RNA was hybridized to double stranded DNA fragments.

Hybridization conditions were identical to those described above, except that the time of hybridization was from 48-72 hours. In addition, after the 55°C wash, the filters were incubated with RNase A (1-2 ug/ml) in 2 X SSC for 5 minutes at room temperature, and then re-washed twice at 55°C in 0.1 X SSC, 0.1% SDS for 15 minutes. The filters were dried and exposed to film as described above.

RESULTS

Level of TK mRNA in various cell types

To examine the steady state levels of TK mRNA in various chicken cells, we compared the TK poly A⁺ RNA content of these cells to the cellular oncogene myc (c-myc) sequence content in the same RNA samples. c-myc mRNA has been reported to be present at approximately 2 - 10 copies/cell in various normal cell types³⁸ and, therefore, represents a fairly reasonable control for the hybridizable quality of our RNA samples, as well as a confirmation of the uniformity of quantitation of our samples. The chicken TK (cTK) probe is a 2.9 kb Hind III fragment isolated from a recombinant DNA plasmid³³ provided by M. Wigler and associates. The chicken c-myc probe is a 3.2 kb Sst I fragment containing both coding exons and an intron, as previously described.^{24,32} As indicated in Figure 1A, both ³²P-nick translated probes detect only one RNA species when hybridized to Northern

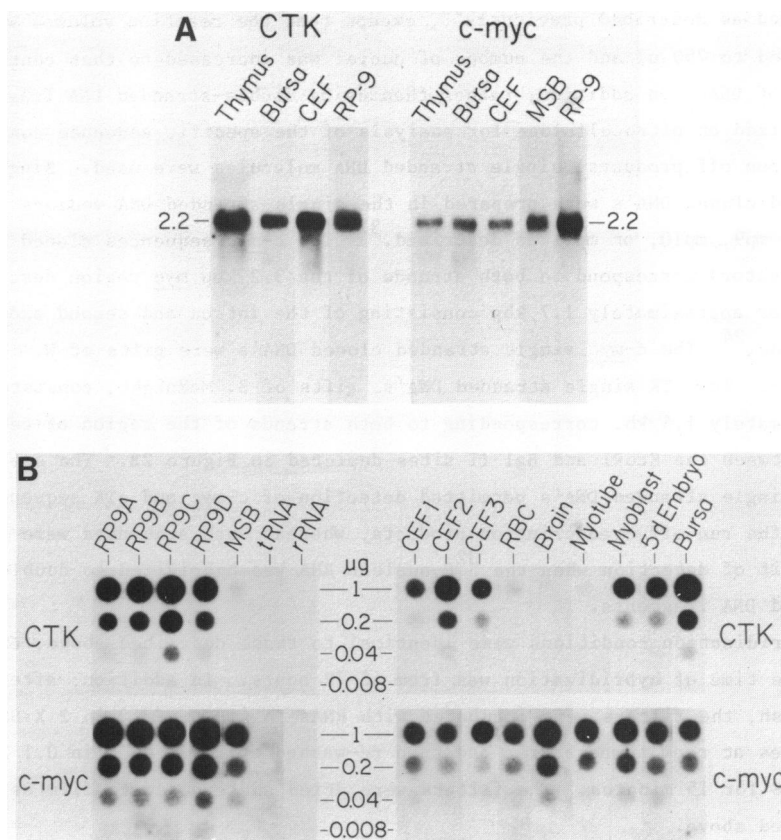


Figure 1: Analysis of *c-myc* and thymidine kinase (TK) sequence content in poly A⁺ RNA from various cell sources. (A) 1.0 ug of poly A⁺ RNA from the indicated cells was run on a formaldehyde gel and transferred to nitrocellulose, and the subsequent "Northern" blots were hybridized to either the *c-myc* probe or the chicken thymidine kinase (cTK) probe. (B) 5-fold serial dilutions of poly A⁺ RNA from the isolated cells were dotted onto nitrocellulose and the resultant "dot-blot" were hybridized to the cTK probe. Subsequently, after removal of the cTK probe by denaturation, the same dot blots were hybridized to the *c-myc* probe.

blots containing poly A⁺ RNA isolated from various cellular sources. Fortuitously, both the cTK and *c-myc* mRNA's are approximately 2.2 kbp in length, as reported by others.^{38,39}

Since both probes detect only a single RNA species by Northern analysis and since both are enriched by poly-A selection (not shown), we used RNA dot blots to compare the cTK and *c-myc* RNA content of various chicken cells.

Poly A⁺ RNA was isolated from total cellular lysates, serial 5-fold dilutions of the RNAs were bound to nitrocellulose filters after denaturation in formaldehyde, and the filters were hybridized to the cTK probe. Subsequently, the ³²P-cTK probe was removed by denaturation and the same filters were hybridized to the c-myc probe. As shown in Figure 1B, neither probe detects ribosomal or transfer RNA sequences, confirming the absence of non-specific hybridization for both probes. To determine the sensitivity of our steady-state assay, we first examined the cTK RNA of several logarithmically growing chicken hematopoietic cell lines with cell cycles ranging from 8-14 hours. Four of these lines (RP9A-D) were derived from transformed B-cells isolated from bursal lymphomas resulting from the insertion of retroviral long terminal repeats (LTR's) within the c-myc locus. These cell lines contain the LTR at various distances from c-myc and all display increased levels of c-myc RNA compared to normal cells.^{24,40} The other cell line, MSB-1, consists of T-cells transformed by Marek Disease virus (MDV), a member of the herpes virus family.^{22,23} MSB-1 cells have a cell cycle time of 8-10 hours, as does RP9C, and lines RP9A, B and D have cell cycles of 12-14 hours. Figure 1b shows that while all RP9 cell lines have significant levels of cTK RNA, MSB-1 cells contain less than 1/25 of the amount of TK RNA compared to the RP-9 lines. As a control, MSB-1 cells contain approximately 5-10 fold less myc RNA as RP9 cells, as expected from the lack of the LTR associated increase in c-myc transcription in these cells. In addition, our results indicate that RP9C cells, which are the fastest growing of the RP9 lines, contain a somewhat higher amount of cTK RNA compared to the slower growing RP9 lines.

The MSB results are rather surprising, given the rapid cell cycle of these cells. One possible explanation is that the cTK gene is no longer active in these cells as a passive result of the presence of viral TK activity. In this view, the cellular gene could have accumulated mutations, become methylated at critical regions, etc., and the cells would still maintain a high rate of division due to the activity of the viral TK gene (see following sections).

We next examined cTK RNA levels in cell cultures and various tissues obtained from normal embryonic and adult chickens. To determine the effect of mitotic activity on the cTK RNA content of cells, we probed RNA from cultures of chick embryo fibroblasts (CEF) containing cells predominately at confluency (CEF1 and CEF3) or in logarithmic (CEF2) growth phase. As shown in Fig. 1B, the confluent culture CEF1 contains approximately 5-10 fold less

TK RNA than CEF2 cells which were harvested in log phase from the same culture as CEF1 by trypsinization and replating. Similarly, a portion of the subcultured CEF 1 cells that were regrown to confluency (CEF 3) also contain 5-10 fold less RNA than their logarithmically growing counterparts. As a control, all three populations contain similar amounts of *c-myc* RNA. Thus, the level of cTK RNA found in CEF appears to be correlated with the proportion of cells in division.

In an attempt to extend these observations, we examined RNA from three populations of post-mitotic cells. Red blood cells (RBC) consisting of 50% immature, transcriptionally active but mitotically inactive erythroblasts were obtained from the peripheral blood of an anemic adult chicken; brain tissue was dissected from a 4 week old hen; and myotubes were derived from pectoralis muscles dissected from an 11 day embryo and cultured as described in Materials and Methods. Fig. 1B shows the results of hybridization of poly A⁺ RNA from these post-mitotic cells to cTK and *c-myc* probes. While these cells contain less than 5% of the cTK RNA present in logarithmically growing CEF, they all contain similar amounts of *c-myc* RNA as that found in the CEF cultures. Thus, the lack of significant levels of steady state cTK poly A⁺ RNA in RBC, brain and myotubes is consistent with the notion that the amount of steady state cTK RNA is correlated with the mitotic activity of a given cell population.

As a additional control for the myotube experiments, we isolated RNA from cultures containing predominantly replicating myoblasts, but some post-mitotic myoblasts and myotubes. Fig. 1B shows that these cultures contain at least 10-20 times the amount of cTK compared to the myotube cultures, but that both cultures contain the same amount of *c-myc* RNA. Similarly, as controls for the uncultured rbc and brain cells, we examined the steady state cTK RNA levels in 5 day embryos, containing a mixture of rapidly dividing and post-mitotic cells including rbc, as well as a highly mitotic population of bursal lymphocytes isolated from the same hen from which the brain tissue was obtained. While both the bursal lymphocytes and 5 day total embryonic RNA contain significantly greater amounts of cTK sequences than any of the post-mitotic samples (Fig. 1B), the *c-myc* RNA content of all of these cells is roughly equivalent.

As is evident in Fig. 1B, bursal lymphocytes and logarithmically growing CEF (CEF2) contain similar amounts of cTK RNA; the myoblasts and 5 day embryonic cells contain approximately 4-5 fold less cTK RNA, and the predominantly confluent CEF (CEF1 and 3) contain somewhat lower levels of cTK

sequences than the myoblasts and embryonic cells. Since we have neither quantitated the mitotic indexes of these cells, nor precisely measured the cell cycle times of each of these cell types, the absolute relationship among cell cycle times, numbers of mitotically active cells and steady state cTK RNA levels can not be derived from these experiments. Clearly, however, these experiments indicate that the relative amount of cTK RNA in given populations of cells is correlated with the divisional activities of such populations.

Methylation of cTK DNA

Since there are a number of reports showing a correlation between hypermethylation and reduced activity of specific cellular genes^{41,42}, as well as data indicating that the methylation of TK sequences in HSV transfectants is associated with the abolition of TK activity in these cells⁴³⁻⁴⁵, we investigated the methylated state of the cTK genes in some of the cells described in the previous section. In this series of experiments, DNA from cells containing high or low amounts of cTK RNA, as well as the generally hypermethylated sperm DNA, were digested with Msp I or Hpa II and blot hybridized to the cTK probe. Msp I and Hpa II are isoschizomers which recognize the tetranucleotide CCGG, but Hpa II will not cleave this sequence if the internal C is methylated.⁴⁶ As shown in Figure 2A, while Msp I digestion and hybridization to the cTK probe reveals a 2.9 kbp fragment in DNA from all of these cells, Hpa II digestion of the same DNA samples reveals several differences in the pattern of methylation among these cell types. Specifically, Msp I and Hpa II digestions reveal the same fragment in RP9 DNA whereas the predominant cTK related Hpa II fragment in brain, bursa and RBC is approximately 100 bases larger than the corresponding Msp I fragment (see below); [the larger bands in the Hpa II digested bursa and RBC DNA are the results of partial digestion (not shown)]. In addition, the cTK gene appears to be methylated at more Hpa II sites in MSB cells than in any of the other DNA samples including sperm DNA.

To determine the specific location of the methylated Hpa II sites in the cTK gene of these various cells, Hpa II digested DNA was re-digested with restriction enzymes that cut within the cTK gene. The results of this analysis are presented in Figure 2B. Neither Eco RI nor Kpn I both of which cut near the 5' end of the gene, result in any change in the size of the fragments generated by Hpa II alone in DNA from RP9, brain, sperm, or MSB cells. However, digestion of these same DNA's with Hpa II and Bgl II, which cuts near the 3' end of the gene, reveals that the major sites of variation in Hpa II methylation in the somatic cells reside in the 3' region outside

the coding portion of the cTK gene, whereas sperm DNA is more highly methylated both in the body of the gene as well as in the 3' non-coding region. This is confirmed by the Hpa II/Pvu II double digestion, since there is a Pvu II site 5' to all of the Hpa II sites in the 3' non-coding region of the gene (Fig. 2B).

Referring to the map at the bottom of this figure, the 5' end of the gene is near the Eco RI site (R) and the poly A addition site is between the Bgl II (B) and Hind III (H) sites.³³ The Msp I sites detectable by blot analysis are represented by the letters a-l on this map. The Bgl II/Hpa II double digests reveals that: (1) Hpa II sites g and h are unmethylated in RP9 cells; (2) in brain, sites g and l are unmethylated, but site h is methylated; (3) sites g, h, and i are fully methylated and sites f and j are partially methylated in sperm DNA; and (4) in MSB cells, site g is unmethylated, whereas sites h, i and j are fully methylated and site k is partially methylated. (For technical reasons, the presence of Hpa II fragments corresponding to these latter sites in MSB DNA are not evident in the Bgl/Hpa II digest, but are observed in the other digests in this figure.) By performing partial Hpa II digests and employing probes for the 5' region of the gene, we have confirmed that Hpa II sites a through g are unmethylated in RP9, brain and MSB DNA (not shown). In addition, RBC, bursal lymphocyte and CEF DNA show patterns of methylation identical to that observed in brain cells (not shown). Thus, the only detectable differences in Hpa II methylation in the cTK gene among these various somatic cells reside outside the coding region and distal to the poly-A addition site of the gene. In particular, even though no steady state cTK RNA is detectable in MSB, brain or RBC cells, and large amounts of cTK RNA is found in RP9 and bursal cells, all of these cells have identical patterns of Hpa II methylation in the coding region of this gene.

Chromatin Structure of cTK

Another possible level of control of cTK expression could reside at the level of chromatin structure. To examine this possibility, we digested isolated nuclei from RP9 and bursal cells which contain large amounts of steady state cTK RNA, and from MSB cells and RBC which contain little, if any, detectable cTK RNA with increasing concentrations of DNase I. The resultant DNA was then restricted with Hind III, electrophoresed on 1% agarose gels and blot hybridized to the cTK probe. The presence and location of cTK associated hypersensitive sites were determined in all of these samples, as were the relative sensitivities of the cTK gene in each cell type.

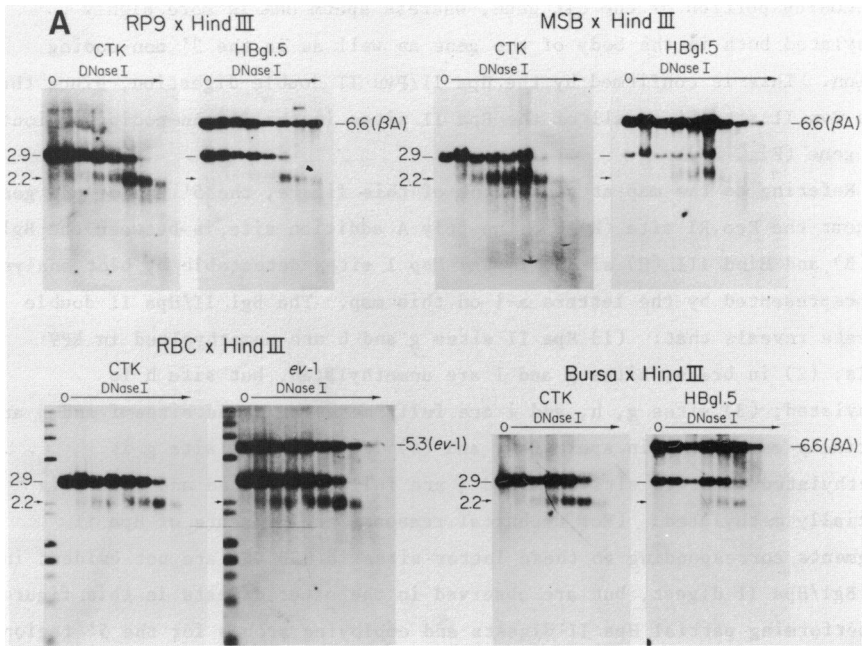


Figure 3: Invariant chromatin structure of the thymidine kinase gene. (A) Nuclei from designated cells were digested with increasing concentrations of DNase I (as designated by the direction of the arrow), DNA isolated, digested with Hind III and blot hybridized to the cTK probe. After autoradiography, the same blots were rehybridized (without removal of the cTK probe by denaturation) to either the adult globin β^A specific probe (HBgl.5) or a probe specific for the endogenous retrovirus (*ev-1*). No globin or *ev-1* subbands are generated by DNase I digestion of these nuclei, when identical blots are probed with HBgl.5 or *ev-1* above, without previous hybridization to cTK. (B) The location of the cTK subband is near the Eco RI site in both red blood cell (A) and bursal lymphocyte (B) nuclei. Representative DNase I digested DNA samples from the bursa and RBC series presented in Figure 3A were digested with either Hind III, Hind III + Bgl II, or Hind III + Eco RI, and blot hybridized to the cTK probe. Since a subband of 1.5 kbp is evident upon Bgl II/Hind III digestion, but not Hind III/Eco RI digestion, the hypersensitive site must be located just to the right of the EcoRI site.

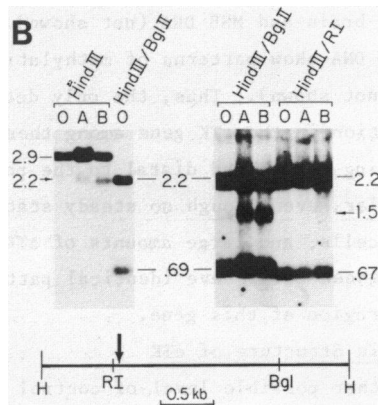


Fig. 3A reveals that a DNase I generated subband of 2.2 kbp is present in all four cell populations, when the corresponding DNA's are analyzed as described above. Fig. 3B shows that the location of the hypersensitive site resulting in the appearance of this subband is similar in DNase I generated DNA sample from either RBC ("A") or bursal lymphocytes ("B"). In both of these samples, as well as in similarly prepared and analyzed samples from RP9, MSB, CEF and brain nuclei (not shown), the 2.2 kb subband is observed upon Bgl II/Hind III double digestion, whereas the subband is eliminated by Eco RI/Hind III double digestion. Thus, in all of these cells, the hypersensitive site is located near the Eco RI site at the 5' end of the cTK gene.

To analyze the relative sensitivities of the cTK gene in these cells, the same blots described above (Fig. 3A) were re-hybridized to genes known to be transcriptionally inactive in these tissues. For ease of comparison of the relative sensitivities of these genes, the cTK probe was not removed prior to re-hybridization to the second probe. For RP9, MSB and bursal cells, an adult (β^A) globin gene probe (HBgl.5)³⁰ was used, and for RBC, a probe specific for an inactive endogenous retrovirus locus (ev-1)³⁵ was used. In all four cases, even though the "inert" gene is represented by a larger restriction fragment than cTK and, therefore, is a larger target for DNase I, it is digested at a slower rate than cTK (Fig. 3A). Thus, by both the criteria of 5' hypersensitive sites and relative sensitivity to DNase I digestion, no differences in cTK chromatin structure are detectable among cells containing high or minimal levels of cTK steady state RNA.

Nuclear Transcription Analysis

In the experiments presented above, no differences in the methylation at Hpa II sites in the coding region of cTK, the DNase I sensitivity of this gene or the presence of hypersensitive sites at the 5' end of cTK are detectable in cells in which either significant or barely detectable amounts of cTK steady state RNA are found. While some of these characteristics of transcriptionally active genes have been described for transcriptionally inert genes, the finding that all three of these parameters are independent of the presence or absence of detectable levels of steady state cTK RNA led us to examine the transcriptional activity of the cTK gene by nuclear runoff transcription. As described previously³⁶, this technique measures the presence of elongating polymerases along a specific gene. Essentially, isolated nuclei are incubated with ³²P-UTP for 5-20 minutes under specific conditions, and the resultant ³²P labelled nascent RNA is hybridized to an

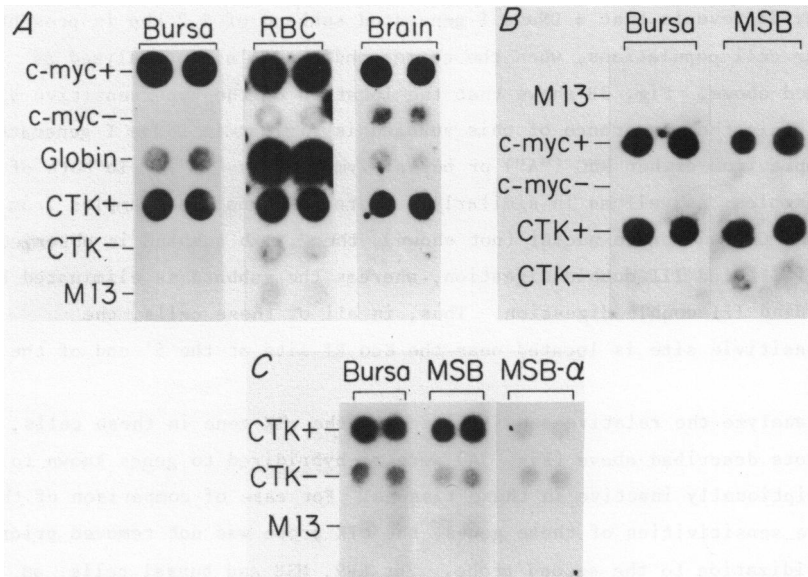


Figure 4: Hybridization of ^{32}P -nuclear RNA runoff transcription products to cloned DNA fragments reveals elongating RNA polymerase molecules along the thymidine kinase gene in nuclei from mitotically active and post-mitotic cells. In A-C, single stranded cloned DNA's containing sequences homologous to the coding (+) and non-coding strands (-) of the chick thymidine kinase gene (cTK) and the c-myc gene, as well as single stranded DNA from the parental M13 vector were immobilized on nitrocellulose, and hybridization to ^{32}P -nuclear runoff products were conducted and analyzed as described in Materials and Methods. In all cases the cTK sequences correspond to the 1.5 kbp between the EcoRI and Bgl II sites represented in the maps in Figures 2B and 3B. (A) The c-myc DNA is derived from the 3.2 kb sequence containing both coding exons and the intron; the globin DNA sequences are double-stranded and are derived from a pBR plasmid containing a 500 bp insert corresponding to intronic sequences of the adult β^A gene (HBg_{1,5}). The low level hybridization to globin observed with bursal ^{32}P -nuclei RNA could be due to RBC containments in the preparation of bursal lymphocytes, or non-specific hybridization to either strands. (B) The c-myc DNA is derived from the 1.7 kb sequence containing the intron and 3' exon of c-myc.²⁴ (C) The non-specific hybridization to the non-coding cTK- strand is not as sensitive to concentrations of α -amanitin (2 ug/ml) which inhibit polymerase II activity, as is the signal from the coding cTK+ strand. The large changes in ratio between the signals observed from the cTK+ and cTK- strands with this concentration of α -amanitin indicate that the hybridization to the cTK+ strand is from polymerase II elongated nascent chains, whereas the hybridization to the cTK- strand is the result of polymerase I or III transcription products.

excess of DNA bound to nitrocellulose. In the case of rarely transcribed genes, such as cTK, we have not been able to detect significant hybridization using double stranded DNA bound to nitrocellulose. However, for reasons that

are not clear at this time, cloned single-stranded DNA maximizes the sensitivity of this assay, permitting the detection of such rarely transcribed genes (M. Groudine, unpublished observations).

Figure 4 shows the amount of cTK nascent transcripts relative to c-myc transcripts in bursal lymphocytes, compared to the amount of these transcripts in either RBC, brain or MSB cells. In Fig. 4A, a 3.2 kbp myc-clone containing two exons and one intron was used, and in Fig. 4B, a 1.7 kbp myc-clone containing the 3' exon and intronic sequences was used for analysis. The single stranded cTK cloned DNA used in all experiments contains 1.5 kbp of coding DNA corresponding to those sequence between the EcoRI and Bgl II sites illustrated in the line drawing in Figure 2B.

As illustrated in Figure 4A, when the c-myc transcription signal is used as a standard for comparison, no significant differences in hybridizable cTK ³²P-RNA are detectable among bursal, RBC or brain nuclei. Thus, RBC and brain nuclei contain as many initiated, elongation competent RNA polymerases along the cTK gene as are present in bursal lymphocytes. In contrast, only ³²P-nascent RNA chains from RBC contain significant amounts of globin (β^A) sequences. Figure 4B shows a comparison between c-myc and cTK transcription in bursa and MSB nuclei. Again, when c-myc transcription is used as a standard, no difference in cTK transcription between bursa and MSB nuclei is found.

The results presented in Figures 4A and 4B are unchanged when the nuclear runoff transcription experiments are conducted in the presence of 0.5% Sarkosyl (data not shown). This compound removes histones and most other chromosomal proteins from chromatin, but leaves initiated polymerases still bound to DNA and capable of elongation, but not new initiation³⁶; thus, in the presence of Sarkosyl, "blocked" RNA polymerases would most likely elongate even if histones or other proteins prevent such such elongation in the intact nuclei. Since Sarkosyl has no effect on cTK transcription in nuclei from any of the cells described above, initiated but blocked polymerases do not appear to be contributing to these results. In addition, no changes in cTK/c-myc transcription ratios are observed during either a 2.5 or 15 minute incubation, indicating that under the conditions of the runoff assay, no significant differences between c-myc and cTK nascent transcript processing are observed.

Comparison of the ratio of c-myc/cTK transcription signals from bursa nuclei shown in Figures 4A and 4B provides support for the notion that c-myc complimentary RNA is synthesized from unique DNA sequences contained in the

c-myc single stranded DNA used in this analysis. For example, since the length of the cTK DNA is constant, the ratio of c-myc/cTK signal should decrease as the length of the c-myc DNA sequence is shortened, unless the bulk of the c-myc signal is derived from repeated transcripts with homology to a portion of the c-myc DNA. In figure 4A, 3.2 kb of c-myc sequences were used and in Figure 4B, 1.7 kb of c-myc sequences were immobilized on nitrocellulose. While the ratio of c-myc/cTK signal from bursal nuclei is approximately 2:1 in 4A, this ratio is 1:1 in 4B. Thus, the observation that the c-myc/cTK signal ratio is dependent on the length of the c-myc sequences bound to nitrocellulose (Figs. 4A and 4B) shows that the observed c-myc signal is representative of hybridization to non-repetitive c-myc transcripts.

In addition, Fig. 4C shows that hybridization to the coding cTK strand (cTK+) is greatly reduced by concentrations of α -amanitin (2 ug/ml) selectively inhibitory for pol II transcription, whereas the variable signal observed from the opposite strand (cTK-) is not as sensitive to inhibition of pol II activity. The same result has been obtained for the c-myc positive and negative strand signals (not shown). Thus, the signal observed from the cTK and c-myc positive strand sequences are also representative of non-repetitive pol II transcription. Finally, additional support for the uniqueness of the c-myc and cTK single stranded DNA's is that double stranded probes containing these same sequences detect only single bands when hybridized to Southern blot containing appropriately digested DNA samples (Figures 2 and 3, and ref. 24).

DISCUSSION

We have observed that the steady state level of poly A+ chicken thymidine kinase RNA is correlated with the mitotic activity of several populations of cells. Post-mitotic rbc, brain and myotubes contain no detectable cTK RNA sequences, and logarithmically growing CEF contain 5-10 fold the amount of cTK RNA as more stationary CEF cultures. Our examination of the chromatin structure and pattern of DNA methylation in the coding region of the cTK gene revealed no differences between cells containing barely detectable levels of cTK RNA and cells containing high levels of this RNA. By employing in vitro runoff transcription of nuclei and subsequent hybridization of the ³²P-nuclear RNA to single stranded DNA fragments containing the cTK coding sequences, we demonstrated that the cTK gene is as transcriptionally active in post-mitotic cells as it is in an actively dividing population of bursal

lymphocytes. Thus, we conclude that the amount of steady state cTK RNA is regulated at the post-transcriptional level.

While many tissue-specific genes appear to be regulated at the level of transcription¹⁻⁷, there have been relatively few investigations into the nature of the controls governing the regulation of ubiquitously expressed genes. Recently, however it was suggested that the principal control of tubulin gene expression in response to altered subunit pools was post-transcriptional in nature, since the rates of transcription of this gene were identical in nuclei from cells containing markedly reduced tubulin mRNA and in control nuclei.⁴⁷ In addition, it has been observed that in growth-stimulated methotrexate-resistant cells containing amplified copies of dihydrofolate reductase (DHFR) DNA and mRNA, the increase in the level of cytoplasmic DHFR mRNA resulted from an increase in the stability of DHFR nuclear transcripts⁴⁸, an observation consistent with post-transcriptional regulation of these genes. More recently, however, it was reported that the content of DHFR mRNA and the relative rate of transcription of the DHFR genes in methotrexate-resistant cells were the same in amino acid starved cultures as in exponentially growing cells.⁴⁹ Our results concerning the transcriptional competence of the cTK gene in normal avian cells no longer active in DNA synthesis are consistent within the notion that ubiquitously expressed cellular genes whose products are involved in DNA synthesis may be regulated post-transcriptionally.

Although neither our observations concerning cTK nor the initial experiments with DHFR address the mechanism of the post-transcriptional regulation of these genes, several possibilities are evident. For example, one possibility could be that poly-adenylation is important in the function of the TK gene product, and that in post-mitotic or stationary phase cells, cTK RNA is present, but not poly-adenylated; however preliminary experiments from our own lab have not revealed the presence of poly A⁻ cTK RNA in a number of growing or post-mitotic cells (C. Casimir, unpublished observation). These preliminary observations would not exclude a mechanism based in the stabilization of these transcription products by alternate poly-A addition sites.⁵⁰ Another class of explanations would include premature termination or attenuation of cTK transcription in the post-mitotic cells. While this possibility will be addressed most directly by determining the ratios of nuclear transcription products from specific regions of the cTK gene in nuclei from dividing and non-dividing cells, the fact that we observed no difference in the cTK/c-myc transcription ratio with inclusion of

Sarkosyl in the runoff experiments argues against the notion that "frozen" or "blocked" polymerases are present along the cTK gene in any of these cells. Another possibility is raised by the observations that the activity of HSV TK is not cell-cycle regulated, whereas cellular TK activities are cell-cycle regulated¹⁸, and that this differential regulation of the viral and cellular genes is based in intragenic rather than promoter sequences (G. Merrill and S. McKnight, personal communication). Since the viral gene does not contain introns⁵¹, the cell cycle regulation of the cellular TK genes could be related to the presence of intronic sequences in the cellular transcripts.³⁹ In this view, processing of the nascent transcript would be required for its stability and accumulation, and this processing event might be dependent upon specific factors, which may be present only at certain times in the cell cycle. Finally, it is also conceivable that the stability of cellular TK RNA is influenced by the presence or absence of the enzyme substrate.

We have also observed that in a rapidly dividing chicken cell line (MSB-1) derived from the Herpes-like virus (MDV) transformation of T-lymphocytes, little if any steady state poly A⁺ cTK is detectable. Our initial hypothesis to explain this finding was that the continued presence of a viral TK gene could have led to the passive loss of expression of the cellular TK gene, possibly via mutation or methylation in the cellular sequences. However, as in the case of the post-mitotic cells, the cTK gene in MSB cells is as transcriptionally active as it is in rapidly dividing cells. This result raises the possibility that a point mutation in cTK sequences important in proper splicing of the nascent transcript may have occurred in these cells, similar to the processing defects observed in some of the human thalassemias.^{52,53} Another explanation for this observation could be that the presence of large quantities of the viral TK product leads to the failure of proper processing of the cellular transcripts, perhaps through some unknown, feedback-inhibition pathway. This latter possibility would predict that appropriately conducted mixing experiments, cell fusions, or permissive infections with the Marek's virus would reveal the same phenomenon, and provide a basis for further investigation of this phenomenon.

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