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TISSUE SPECIFIC METHYLATION OF C-MYC IN ADULT CHICKENS

C. Heinke, L. Andersen, S. Hagan and K.S. Guise

Department of Animal Science
University of Minnesota
St. Paul, Minnesota 55108

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Abstract. Methylation of cytosine in DNA has long been correlated with modulation of specific gene expression in eukaryotes. Methylation of the c-myc locus was examined in six tissues from adult Leghorn chickens. The c-myc locus was found to be variably methylated in all examined tissues, except blood, where erythrocytic DNA showed no evidence of significant methylation of c-myc. This is contrasted with the observed severe methylation of the ß actin locus and the generally high methylation patterns found in avian erythrocytic DNA.

Methylation of DNA has been repeatedly correlated with modulation of specific eukaryotic gene expression (1), possibly by altering the conformation of DNA to adopt the left handed Z-DNA form (2). DNA methylation has also been shown to be the basis for more generalized gene inactivation events such as the Lyonization of the X chromosome in female mammals (3). Actively transcribing genes are generally thought to be under or hypomethylated (4). Methylation of cytosine to produce 5-methylcytosine, has been found almost exclusively in the sequence $^{5'}CG^{3'}$, where 50-70% of all such dinucleotides were modified by methylation in both mammalian and avian species (5). DNA methylation patterns have subsequently been implicated in a major role in differential gene expression in higher eukaryotes (6).

Cellular oncogenes, so named from their original identification by homology with oncogenic viral sequences, are responsible for a variety of basic growth regulatory functions during development and differention. Phylogenetic conservation of these genes in metazoa lends credence to this theory. The gene c-myc represents the cellular locus that is homologous to the oncogene of the avian myelocytomatosis virus (MC29). MC29 is responsible for wide range of malignant avian diseases including myelocytomas, carcinomas, sarcomas and erythroblastosis (7). Avian leukosis virus (ALV) has been shown to be a causative agent of lymphoid leukosis, characterized by B-cell lymphomas. Integration of the ALV

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provirus near the cellular oncogene c-myc has been observed in a majority of tumors, with a concomitant elevation in the expression of c-myc (8,9).

Avian c-myc encodes a 58 kilodalton phosphoprotein (10) whose function has yet to be determined. The purpose of this study is to investigate the possible functions of c-myc by assaying the state of methylation of the c-myc locus in various adult chicken tissues, as evidence of current or recent gene activity.

MATERIALS AND METHODS

Avian Tissue Preparation: Blood was drawn from the brachial vein of adult White Leghorn male chickens into syringes containing 0.1 ml of 0.1 M EDTA per 5 mls of collected blood. Samples were centrifuged at 200 g for 10 minutes at 25°C. The erythrocyte pellets were washed by addition of one volume of 1 x SSC (0.15 M NaCl, 0.015 M NaCitrate), and stored at -20°C or -80°C. Liver, heart, brain, gizzard and breast muscle were removed from birds immediately upon sacrifice, wrapped in aluminum foil, immersed in liquid nitrogen until frozen, and stored at -80°C.

DNA Isolation from Erythyrocytes: 100 µl of the thawed erythrocyte pellet were resuspended in 2.0 mls HTE (100 mM Tris · HCl, pH 8.0, 40 mM EDTA). Cells were lysed by addition of 2.0 mls of 0.2% sodium dodecyl sulfate (SDS) in HTE. An equal volume of phenol saturated with 10 mM Tris · HCl, pH 8.0, 1 mM EDTA and 0.2% ß-mercaptoethanol was added, and the sample vigorously mixed. The resultant emulsion was centrifuged at 480 g for 10 min at 4°C. The aqueous phase was extracted twice with saturated phenol, and once each with 2.0 mls of chloroform:isoamyl alcohol (24:1), and 2.0 mls of anhydrous ether. One-tenth volume of 4.0 M ammonium acetate was added to the aqueous phase and the sample mixed by inversion. DNA was precipitated by addition of two volumes of absolute ethanol followed by overnight storage at -20°C. The sample was microfuged at 12,100 g for 10 min at 4°C and the pellet dried *in vacuo*. The dried pellet was resuspended in 50 µl LTE (10 mM Tris · HCl, pH 8.0, 1 mM EDTA) and DNA concentration determined spectrophotometrically.

DNA Extraction from Tissues: Approximately 1 g of frozen tissue was minced by polytron for 1-3 minutes on ice in 1 ml/g of PBS, pH 7.4 (phosphate buffered saline:0.0015 M KH_2PO_4, 0.14 M NaCl, 0.016 M Na_2HPO_4, 0.0027 M KCl). 10 ml of the extraction buffer (0.1 M EDTA, pH 8.0, 100 µg/ml pronase, and 0.5% SDS) were added, the solution vortexed and incubated 2 hrs at 37°C. An equal volume of LTE saturated phenol:chloroform (1:1) was added. The sample was mixed by inversion, and centrifuged at 480 g for 10 min at 4°C. The phenol extraction was repeated once. The aqueous phase was adjusted to 0.25 M NaCl by addition of 5 M NaCl. Two volumes of cold 100% ethanol (-20°C) was added to the solution, and the DNA spooled onto a glass rod and air dried. The DNA was resuspended in 2.0 mls LTE, pH 8.0, and concentration determined spectrophotometrically.

DNA Analysis: DNA samples were digested with either restriction endonuclease MspI or HpaII (20 units/10 µg DNA, Bethesda Research Labs) overnight at 37°C in 10 mM TRIS·HCl, pH 7.5, 10 mM.$MgCl_2$, 2 mM dithiothreitol. The cleaved DNA was subjected to submarine agarose gel electrophoresis on a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide for 18-24 hours at 35 volts in TBE buffer (90 mM Tris-base, 2 mM EDTA, 90 mM Boric acid). Samples from three individual birds were examined concurrently on the same gel. Analysis was repeated to confirm complete digestion of DNA samples. The gels were either subjected to Southern transfer onto BA85 nitrocellulose (11) or alkaline Southern transfer onto Genescreen Plus (New England Nuclear). Alkaline transfer used a 30 min denaturing presoak in 0.4 M NaOH, 0.6 M NaCl, an overnight transfer using the same denaturing buffer, followed by washing of the Genescreen filters in 0.5 M Tris·

HCl, pH 7.0, 1 M NaCl for 15 min. BA85 filters were baked at 80°C for 2 hours in vacuo, Genescreen filters were air-dried.

BA85 filters were prehybridized 48 hours at 43°C in 0.2 ml/cm^2 prehybridization buffer:3X SSC (1X SSC = 0.15 M NaCl, 0.015 M NaCitrate), 1X Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% ficoll), 0.05 M Na phosphate, pH 6.8, and 150 µg/ml denatured salmon sperm DNA. A 1560 basepair v-myc probe from MC29 (Oncor, Gaithersburg, MD) was nick-translated with [^{32}P] dCTP to a specific activity of 1-2 x 10^8 cpm/µg using protocol and kit supplied by Amersham. 0.1 µg of probe in 0.05 ml/cm^2 of prehybridization buffer containing 10% dextran sulfate was hybridized to the filter at 43°C for 48 hrs. Filters were washed at 65°C in 3 sequential wash solutions A: 0.05 M Tris·HCl, pH 8.6, 0.002 M EDTA, pH 8.4, 1.0% SDS and 1.0 M NaCl; B: 0.5% SDS, 0.025 NaPhosphate, pH 6.8, 0.5 M NaCl; and C: 0.5% SDS, 0.05 M Tris HCl, pH 8.6, 0.5 M NaCl; for 1 hour each solution with 2 changes. Filters were briefly air-dried and exposed to Kodak XAR-5 film for 7 days at -80°C with intensifying screens.

Genescreen Plus filters were prehybridized with 50 ml of 1% SDS, 1 M NaCl, 10% Dextran sulfate, 0.05% NaPyrophosphate, and 100 µg/ml denatured salmon sperm DNA for 24 hrs at 75°C. Hybridization was with 25 mls of fresh prehybridization buffer plus 0.2 µg of nick translated (1-2 x 10^8 cpm/µg) 770 bp chicken ß-actin cDNA probe (Oncor) at 75°C for 48 hours. Filters were washed sequentially for 60 min in A: 2X SSC, 0.05% NaPyrophosphate, 0.1% SDS at 25°C; B: 2X SSC, 0.05% NaPyrophosphate, 0.1% SDS at 75°C; and C: 0.1X SSC, 0.05% NaPyrophosphate at 75°C. Filters were exposed to Kodak XAR-5 film for 3 days at -80°C using intensifying screens.

RESULTS: Erythrocytic DNA, whether digested with MspI (methylation insensitive) or HpaII (methylation sensitive) revealed one intense autoradiographic band at approximately 2.5 kb when probed with v-myc (Figure 1). Digests of liver, brain, gizzard, heart, and breast muscle also revealed the heavy 2.5 kb band with either enzyme, but HpaII digests generated additional bands of higher

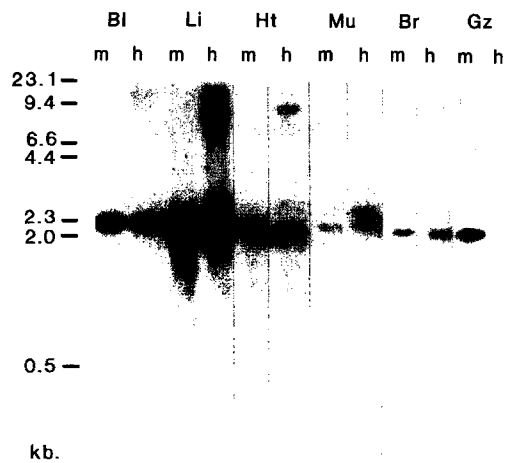

Figure 1. Autoradiographic pattern of white leghorn genomic DNA from blood (Bl), liver (Li), heart (Ht), muscle (Mu), brain (Br), and gizzard (Gz) after digestion with MspI (m) or HpaII (h) and probing with a 1560 bp V-myc probe.

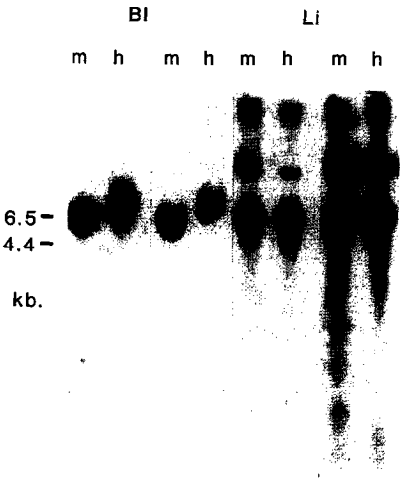

Figure 2. Autoradiographic patterns of white leghorn genomic DNA from blood (Bl) and liver (Li) after digestion with MSPI (m) or HpaII (h) and probing with a chicken ß actin 770 bp cDNA.

molecular weight in all five tissues, indicating existence of partially-methylated sequences (Figure 1). By contrast, autoradiographs of blots probed with chicken ß-actin showed little methylation-related difference between MspI and HpaII digests of liver DNA. However, there was major methylation of the actin locus in erythrocytes as evidenced by the approximately 1.3 kb shift in the 5.0 kb major actin band (Figure 2).

DISCUSSION: That c-myc would show a lack of significant methylation in erythrocytes was unexpected since erythrocytic DNA is in general highly methylated. This heavy methylation can be visualized in the skewed of the ethidium bromide patterns of DNA digested with HpaII compared with those of DNA digested with the isoschizomer MspI. The same CCGG sequence is recognized by MspI and HpaII but unlike HpaII, MspI will cleave if the internal cytosine is methylated. The ß-actin methylation described in this paper shows the major methylation of that locus in erythrocytes. C-myc, while showing partial methylation in all the non-erythrocytic tissues assayed, exhibited no significant methylation at the MspI/HpaII sites in erythrocytes. Given the currently recognized correlation of gene expression or activity with the lack of methylation, this may indicate that c-myc is active in the later stages of hematopoiesis. This would be consistant with the observed correlation of increased levels of c-myc mRNA with increased rate of cell division (12). C-myc mRNA is quite unstable with a half-life of 10 to 20 min. (13). The c-myc protein half-life is similarily short, 20 to 30 min. (14). This rapid protein and mRNA turnover would require the continued synthesis of new c-myc mRNA in the very last stages of hematopoiesis, if indeed c-myc is necessary for mitotic proliferation. Our results showing near major

demethylation of MspI/HpaII sites in and near the c-myc locus is consistant with the proliferative function of the c-myc gene. The actin locus, also very necessary for cell proliferation but producing a more stable mRNA and an extremely stable protein, is extensively methylated in the mature erythrocyte. This raises the question of why the c-myc gene in chicken erythrocytic DNA exists in an apparently active state, since mature mammalian erythrocytes function without the product of the c-myc locus. Indeed mammalian erythrocytes must function without the aid of c-myc due to the short half-life of c-myc mRNA and protein, and the lack of non-degraded DNA in the mature mammalian erythrocyte for new c-myc mRNA synthesis. Although chicken erythrocytic DNA has been thought to be non-functional, in part due to heavy methylation, this assumption should be reexamined in the light of our observations of the c-myc locus.

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