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Developmental and Tissue-Specific Expression of U4 Small Nuclear RNA Genes

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U4 RNA is one of several small nuclear RNAs involved in the splicing of mRNA precursors. The domestic chicken has two genes per haploid genome that are capable of encoding U4 RNA. The U4X RNA gene (which encodes a sequence variant of U4 RNA that was unknown prior to the cloning of the gene) and the U4B RNA gene were both expressed in vivo in each of seven adult and three embryonic chicken tissues examined. However, the ratio of U4B RNA to U4X RNA can vary more than sevenfold in both a tissue- and stage-specific manner.

The small nuclear RNAs (snRNAs) of the U family (U1 to U10) are evolutionarily conserved and metabolically stable RNA molecules present in the nuclei of eucaryotic cells (16). The U1, U2, U4, U5, and U6 snRNAs are involved in the splicing of mRNA precursors (14, 18, 19). In metazoans, there are multiple copies of the genes for each of the major snRNAs (usually 5 to 1,000 gene copies per family) (3). However, in the chicken there are only two genes per haploid genome that are capable of encoding U4 RNA (6). The organization of the U4X and U4B RNA genes in the chicken genome is diagrammed at the top of Fig. 1. The U4B and U4X genes are closely linked and are in the same transcriptional orientation (6). The sequences of the encoded RNA transcripts are shown at the bottom of Fig. 1. The U4B and U4X RNAs differ in sequence at seven nucleotide positions. U4B is a major U4 RNA sequence variant that has been identified in chickens and other vertebrates (8, 16), but the U4X RNA transcript has not previously been characterized.

Differential accumulation of U4B and U4X RNA in adult tissues. To study the differential accumulation of these U4 snRNAs, we synthesized two DNA 19-mer oligonucleotides (Fig. 1, U4B probe and U4X probe) that were specifically complementary to the 3′ ends of U4B RNA and U4X RNA, respectively. To examine the relative accumulation of U4B and U4X transcripts in various tissue types, we isolated total cellular RNA from seven tissue samples from an adult hen by using the guanidinium-hot phenol method described by Maniatas et al. (13). Purified RNA was dissolved in 99% formamide, run on a 6% polyacrylamide–7 M urea gel, and electroblotted to GeneScreen Plus (Du Pont, NEN Research Products) at 60 V for 1 h and then at 30 V for 45 min, with a chamber buffer consisting of 12 mM Tris acetate, 6 mM sodium acetate, and 0.3 mM EDTA (pH 7.5). The filter was prehybridized at 45°C in 0.9 M NaCl–1% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.6)–0.5 mM EDTA–8 μg of *Escherichia coli* DNA–20 μg of ATP per ml. After 4 h, radiolabeled U4B probe (>20 × 10^6 cpm, labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase) was added, and hybridization was continued for 2 h. Following hybridization, the filter was rinsed twice (each time for 1 h in 0.3 M NaCl–0.03 M sodium citrate [pH 7]–1% sodium dodecyl sulfate at 45 to 50°C) and autoradiographed.

The resultant autoradiogram is shown in the upper panel of Fig. 2. The amount of RNA loaded in each lane was adjusted (based on results from preceding blots) to give a signal of uniform intensity when hybridized to the ^{32}P-labeled U4B probe. Following autoradiography, the U4B probe was eluted from the filter by two 1-h washes in distilled water at 75°C. The same filter was then rehybridized to the radiolabeled U4X probe (autoradiogram shown in Fig. 2, lower panel). To ensure the hybridization specificity of the U4B and U4X probes for the respective U4 RNA variants, artificial U4X-like (SP6:U4X) and U4B-like (SP6:U4B) transcripts, approximately 178 and 233 nucleotides in length, respectively, were included in the gels as internal controls. These were generated by transcribing the cloned genes in vitro with SP6 RNA polymerase as specified by the supplier (Promega). Relevant portions of the templates (cloned into the pSP64 vector) are diagrammed in Fig. 1.

U4B RNA and U4X RNA were both present in all seven of the adult tissues examined (Fig. 2). However, their relative level of accumulation differed from one tissue to another, as reflected by the variation in intensity of the U4X signal (lower panel). Among the seven tissues examined, the ratio of U4X to U4B RNA was lowest in gizzard and oviduct tissues, somewhat higher in breast muscle and kidney tissues, still higher in brain tissue, and at its highest level in heart and liver tissues. A quantitation of the actual U4X-to-U4B RNA ratios in two of these tissues (kidney and gizzard) is addressed in experiments described below.

Differential accumulation of U4B and U4X RNAs during development. We next investigated whether the accumulation of the U4B and U4X RNA gene products is differentially regulated during embryonic and postembryonic development. RNA was isolated from the brain, gizzard, and heart of 10-day embryos, 17-day embryos, a juvenile chicken (16 days posthatching), and an adult hen. These RNA samples were then subjected to Northern (RNA) analysis as above. The filter was hybridized first with the radiolabeled U4B probe (Fig. 3, upper panel) and subsequently with the radiolabeled U4X probe (lower panel). In each of the three tissues, a reduction in U4X expression (relative to the normalized U4B signal) was observed as development proceeded from the 10-day embryo to the adult. Notably, however, the pattern was programmed differently in each tissue with respect to timing and magnitude. In brain tissue, the 10-day embryonic U4X signal was relatively weak, and

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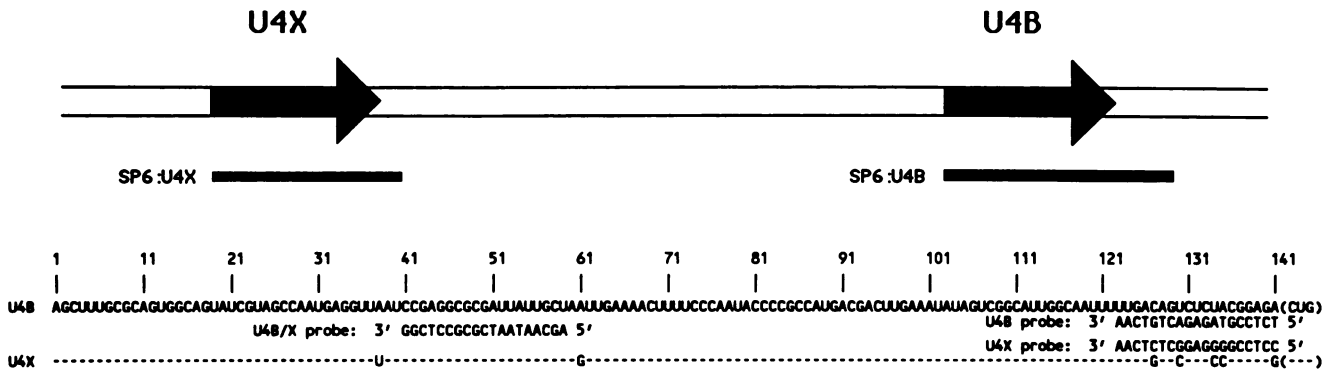

FIG. 1. Genomic organization of chicken U4 RNA genes and sequences of their corresponding RNA transcripts. The U4X and U4B RNA genes are closely linked in the chicken genome and are depicted in the figure by bold arrows pointing in the direction of transcription (6). The second line shows the locations of two restriction fragments that were cloned into the pSP64 vector and were transcribed with SP6 RNA polymerase to generate artificial U4X and U4B transcripts (SP6:U4X and SP6:U4B), which were included as internal controls on the Northern blots. The U4B RNA and U4X RNA sequences encoded by the two genes are shown at the bottom of the figure. In the lower line, dashes indicate sequence indentity between the two U4 RNA gene products, whereas base differences are shown explicitly. The parentheses enclosing the last three nucleotides indicate possible length heterogeneity at the 3' end of U4 RNA encompassing these positions (16). Also shown are the sequences of three synthetic DNA oligonucleotides (U4B/X probe, U4B probe, and U4X probe) complementary to specific regions of the U4B and/or U4X RNAs as indicated by their alignments along the RNA sequences.

there was only a minor reduction in intensity at later developmental stages. In contrast, the U4X signal was quite intense in RNA isolated from 10-day embryonic gizzard tissue, but there was a striking decrease, relative to U4B levels, in RNA samples from later developmental stages. During the same period of development, the heart likewise exhibited a unique pattern of U4X to U4B RNA expression. In summary, our data reveal that the U4X-to-U4B RNA ratio generally decreases as development proceeds, but the rate of the decrease and the final U4X-to-U4B RNA ratio in a particular adult organ is regulated in a tissue-specific manner.

Quantitation of relative amounts of the U4B and U4X RNAs in adult and embryonic tissues. Actual U4X-to-U4B RNA ratios cannot be deduced from the above experiments, because of differences in the specific activities of the U4B and U4X probes and possible differences in their hybridization efficiencies. To determine the percentage of the total U4 RNA corresponding to U4B RNA and the percentage corre-

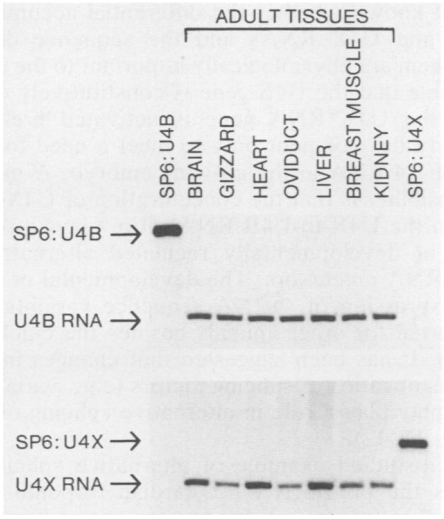

FIG. 2. Northern blot analysis comparing the expression of U4B and U4X RNAs in adult chicken tissues. Total cellular RNAs were isolated from seven tissues of a single adult hen, electrophoresed in separate lanes of a polyacrylamide gel, and transferred to a GeneScreen Plus membrane filter. The two panels shown represent the same Northern blot filter hybridized first to the radiolabeled U4B probe (upper panel) and subsequently to the radiolabeled U4X probe (lower panel). Amounts of RNA loaded (0.15 to 0.70 A_{260} units per lane) were normalized to give signals of uniform intensity when hybridized with the U4B probe. Artificial U4B and U4X transcripts (SP6:U4B and SP6:U4X, synthesized by using SP6 RNA polymerase) were included as internal controls to ensure the hybridization specificity of the U4B and U4X 19-mer probes. The lack of a signal at the position of the SP6:U4B transcript in the lower panel also indicates the efficiency of U4B probe removal from the filter prior to hybridization with the U4X probe.

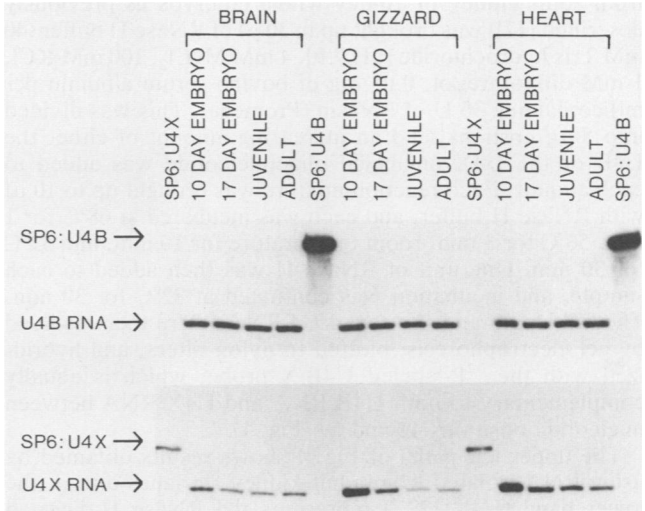

FIG. 3. Northern blot analysis comparing the expression of U4B and U4X RNAs during development. Northern analysis was performed as described in the legend to Fig. 2, except that total cellular RNAs isolated from the brain, gizzard, and heart of 10-day embryos, 17-day embryos, a juvenile chicken (16 days posthatching), and an adult hen were used. Upper panel: hybridization to the U4B probe; lower panel: subsequent hybridization of the same filter to the U4X probe.

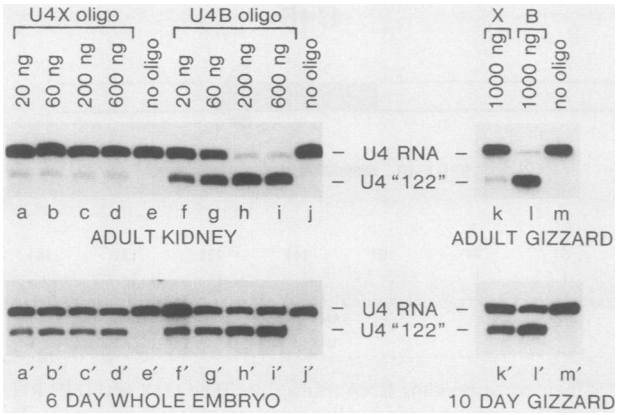

FIG. 4. Northern blots to determine the percentages of U4X and U4B RNAs in adult and embryonic chicken tissues. In the upper left panel, nuclear RNA prepared from adult kidney was hybridized to increasing amounts of either the unlabeled U4X oligonucleotide probe (lanes a to d) or the unlabeled U4B oligonucleotide probe (lanes f to i) and then digested with RNase H. The samples in the control lanes (lanes e and j) were treated with RNase H but contained neither oligonucleotide. The bands labeled U4 "122" represent the shortened U4X RNA (in lanes a to d) and U4B RNA (in lanes f to i). Upper bands represent undigested U4 RNA. The digested and undigested U4 RNAs were detected on the Northern filter by hybridization to the ^{32}P-labeled U4B/X probe shown in Fig. 1. In the lower left panel, an analogous experiment was performed, except that nuclear RNA prepared from 6-day whole embryos was used. In the right-hand panels, total cellular RNA (10 μg) from adult gizzard (upper panel) or 10-day embryonic gizzard (lower panel) tissue was hybridized to a saturating concentration (1,000 ng) of either U4X probe (lanes k and k') or U4B probe (lanes l and l') or to no oligonucleotide (lanes m and m') prior to RNase H digestion and Northern blot analysis.

sponding to U4X RNA in selected tissues, we used a different but related procedure. In the experiments shown in the two left panels of Fig. 4, 60 μg of nuclear RNA (prepared from adult kidney or 10-day whole embryos as previously described [17]) was brought up in 30 μl of RNase H buffer (40 mM Tris hydrochloride [pH 7.9], 4 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 0.03 mg of bovine serum albumin per ml) containing 30 U of RNasin (Promega). This was divided into 5-μg portions, and an increasing amount of either the U4B or the U4X unlabeled oligonucleotide was added to each aliquot. Each reaction mixture was brought up to 10 μl with RNase H buffer, and each was incubated at 68°C for 1 min, 56°C for 3 min, room temperature for 10 min, and 32°C for 30 min. One unit of RNase H was then added to each sample, and incubation was continued at 32°C for 30 min. The full-length and shortened U4 RNAs were then resolved by gel electrophoresis, blotted to nylon filters, and hybridized with the ^{32}P-labeled U4B/X probe, which is equally complementary to both U4B RNA and U4X RNA between nucleotide positions 41 and 59 (Fig. 1).

The upper left panel of Fig. 4 shows results obtained by using RNA isolated from adult kidney. In lanes a to d, the lower band (U4 "122") represents the RNase H-digested U4X RNA, whereas in lanes f to i, the lower band corresponds to RNase H-digested U4B RNA. At saturating amounts of unlabeled oligonucleotide (lanes a to d and h to i), the intensities of the bands directly reflect the relative amounts of U4B RNA and U4X RNA in the sample. Importantly, the U4X and U4B probes yielded results that complemented each other very precisely with respect to band intensities. It is apparent that in adult kidney tissue, U4X RNA accounts for only a minor fraction of the total U4 RNA present. When the experiment was performed with RNA prepared from 6-day whole embryos, U4X RNA was found to be a significantly higher proportion of the total (Fig. 4, lanes a' to j'). To ascertain the actual percentages, individual bands corresponding to U4X RNA and to U4B RNA were cut out of the filters and quantitated by scintillation counting. The results (not shown) indicated that the composition of U4 RNA in adult chicken kidney tissue was about 15% U4X RNA and 85% U4B RNA, whereas in 6-day embryos the composition was about 35 to 40% U4X and 60 to 65% U4B RNA.

The two smaller panels on the right side of Fig. 4 show the results of a similar analysis of U4X RNA and U4B RNA percentages in adult gizzard tissue and in 10-day embryonic gizzard tissue, a tissue which exhibited a wide variation in relative expression levels of the two gene products during development (Fig. 3). The autoradiograms and results from scintillation counting revealed that U4X RNA represented a very minor fraction (7%) of the U4 RNA in adult gizzard, but a significantly higher proportion (35%) in 10-day embryonic gizzard.

Summary and conclusions. Our results show that the relative accumulation of the chicken U4B and U4X RNA gene products is regulated in a developmental and tissue-specific manner. Among the tissues examined, the U4X-to-U4B RNA ratio varied as much as sevenfold (from 1:1.8 in 10-day embryonic gizzard to 1:13 in adult gizzard). At intermediate stages of development and in other tissues examined, the U4X-to-U4B RNA ratios were within this range of values.

It is not known whether the differential accumulation of the U4B and U4X RNAs and the sequence differences between them are physiologically important to the organism. It is possible that the U4B gene is constitutively expressed and that the U4X RNA gene is activated preferentially during early development only to meet a need for a larger amount of U4 RNA in the growing embryo. A more interesting possibility is that the concentration of U4X RNA, or changes in the U4X-to-U4B RNA ratio, plays a role in the selection of developmentally regulated alternative splice sites in mRNA precursors. The developmental or cell type-specific expression of snRNA sequence variants has also been reported for other animals besides the chicken (4, 7, 10–12, 15). It has been suggested that changes in the concentrations or ratios of splicing factors (e.g., variants of the snRNPs) may play a role in alternative splicing of mRNAs (1, 4, 5, 9, 11, 12).

One well-studied example of alternative splicing in the chicken is the pre-mRNA for cardiac troponin T (2). A signficant change in its splicing pattern occurs between days 10 and 18 of embryonic development (2). Interestingly, this shift in the splicing pattern of troponin T correlates temporally with a substantial reduction in the U4X-to-U4B RNA ratio observed in the heart during the same period of development (Fig. 3). A determination of whether this correlation is mechanistically relevant requires further experimentation.

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