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Structural and Functional Analysis of Chicken U4 Small Nuclear RNA Genes

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Two distinct chicken U4 RNA genes have been cloned and characterized. They are closely linked within 465 base pairs of each other and have the same transcriptional orientation. The downstream U4 homology is a true gene, based on the criteria that it is colinear with chicken U4B RNA and is expressed when injected into *Xenopus laevis* oocytes. The upstream U4 homology, however, contains seven base substitutions relative to U4B RNA. This sequence may be a nonexpressed pseudogene, but the pattern of base substitutions suggests that it more probably encodes a variant yet functional U4 RNA product not yet characterized at the RNA level. In support of this, the two U4 genes have regions of homology with each other in their 5′-flanking DNA at two positions known to be essential for the efficient expression of vertebrate U1 and U2 small nuclear RNA genes. In the case of U1 and U2 RNA genes, the more distal region (located near position −200 with respect to the RNA cap site) is known to function as a transcriptional enhancer. Although this region is highly conserved in overall structure and sequence among U1 and U2 RNA genes, it is much less conserved in the chicken U4 RNA genes reported here. Interestingly, short sequence elements present in the −200 region of the U4 RNA genes are inverted (i.e., on the complementary strand) relative to their usual orientation upstream of U1 and U2 RNA genes. Thus, the −200 region of the U4 RNA genes may represent a natural evolutionary occurrence of an enhancer sequence inversion.

The small nuclear RNAs (snRNAs) U1, U2, U3, U4, etc., are a special class of metabolically stable RNA molecules present in the nuclei of eucaryotic cells. The snRNAs are synthesized by RNA polymerase II (11, 32, 36, 44), but they are not polyadenylated and have an unusual 2,2,7-trimethylguanosine cap structure. In vivo they are associated with proteins as components of small nuclear ribonucleoprotein particles (snRNPs). Considerable evidence now exists that U1 and U2 snRNPs play a role in the splicing of mRNA precursors (3, 8, 22, 23, 35, 39). The U5 snRNP has also been implicated in pre-mRNA splicing (5), whereas the U7 snRNP is involved in the maturation of the 3′ ends of histone mRNA (2, 50). U4 and U6 snRNAs coexist in the same snRNP particle (4, 19), and recent evidence indicates that the U4/U6 snRNP is also involved in pre-mRNA splicing (1a, 3a).

Our laboratory is interested in studying the molecular mechanisms of snRNA gene expression. We have previously reported the characterization of four chicken U1 RNA genes (10, 44) and one chicken U2 RNA gene (21a). As with other snRNA genes, chicken U1 and U2 RNA genes lack the two promoter elements most commonly associated with genes transcribed by RNA polymerase II, the so-called TATA and CCAAT boxes. Nevertheless, two distinct and evolutionarily conserved regions of homology are observed in the 5′-flanking DNA of most vertebrate snRNA genes (1, 10, 21a, 25, 29, 48, 51, 53–55, 57). The proximal region (centered near nucleotide position −55 with respect to the RNA cap site) is required for accurate initiation of snRNA transcription (7, 48) and apparently is functionally equivalent to a TATA box. The distal conserved region is located approximately 200 base pairs (bp) upstream from the cap site and has properties similar to those of a classical enhancer element (1, 25, 27, 31). Alterations in either of these conserved regions significantly decrease the transcriptional activity of human and frog U1 and U2 RNA genes assayed by injection into *Xenopus laevis* oocytes (7, 25, 31, 48, 54).

To more fully understand the role of *cis*-acting elements in the control of chicken snRNA gene expression, we have cloned and sequenced two full-length U4 homologies from the chicken genome. One of these homologies is a true gene that codes for chicken U4B RNA (nomenclature of Reddy [41]). The functional status of the second gene is unknown. An analysis of the 5′-flanking DNA of these two U4 RNA genes reveals that they have some features in common with each other and with the controlling elements of other vertebrate snRNA genes; however, some aspects of U4 RNA promoter structure appear to be unique, or altered, relative to the U1 and U2 RNA genes.

MATERIALS AND METHODS

Isolation of genomic clones. Lambda phage clones containing sequences coding for U4 RNA were isolated from a chicken genomic library provided by C. Hodgson, M. J. Tsai, and B. W. O'Malley. This library was constructed with partially *Mbo*I-digested fragments of hen oviduct DNA cloned into the polylinker *Bam*HI site of the lambda phage vector EMBL-4.

The library was screened with a cloned human U4 RNA pseudogene sequence. The pseudogene clone, containing 68 bp of U4 homologous sequence inserted into the *Pst*I site of pBR322, was originally derived from the U4/5 locus (18). S. Berget provided us with this subclone. Following double digestion of the plasmid with *Eco*RI and *Hin*fI, the 1.2-kilobase (kb) fragment containing the U4 sequences was purified on a 1.8% agarose gel. It was then nick translated and used as a hybridization probe. Approximately 500,000 phage plaques (~7 chicken genome equivalents) were screened under the following hybridization conditions: 0.9 M NaCl, 0.09 M sodium citrate (pH 7), 0.04% bovine serum albumin, 0.04% polyvinylpyrrolidone, 0.04% Ficoll, 0.5% sodium dodecyl sulfate (SDS), and 1 mM EDTA, at 50°C.

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Following three rounds of plaque purification, five intensely hybridizing clones were isolated and designated λU4-6, λU4-31, λU4-40, λU4-41, and λU4-47. Subsequent restriction enzyme analysis indicated that clones λU4-6 and λU4-47 were identical, as were λU4-31 and λU4-41. Therefore further experiments used only phage clones λU4-6, λU4-40, and λU4-41.

Detailed analysis of these three clones revealed that they produced similar patterns after restriction enzyme digestion. Furthermore, Southern blots indicated that the U4 probe hybridized to a single 2.1-kb *Bam*HI fragment in each clone. This 2.1-kb fragment from λU4-6 was subcloned into plasmid pBR322, and the resulting plasmid was designated p2.1(λU4-6). Finer restriction mapping and Southern analysis of this plasmid revealed that the U4 probe hybridized to two separate subregions within the 2.1-kb *Bam*HI fragment. The exact location of the U4 homologies within the two hybridizing regions of the 2.1-kb fragment were subsequently determined by DNA sequence analysis.

DNA sequencing. DNA sequence analysis was done by both the chemical degradation method of Maxam and Gilbert (33) and the enzymatic termination method of Sanger et al. (46). In the chemical degradation procedure, restriction fragments were 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP or 3′-end labeled with DNA polymerase I (Klenow fragment) and an α-^{32}P-labeled deoxynucleotide triphosphate. For the Sanger dideoxy protocol, restriction fragments were cloned into the polylinker region of M13mp18 and M13mp19. The dideoxy-terminated fragments were uniformly labeled during synthesis using deoxyadenosine 5′-[α-^{35}S]thio-triphosphate. Complete data were obtained for both strands of the 1,665 bp of DNA sequence presented in Fig. 2.

Oocyte nuclear injections and RNA analysis. In initial experiments, the plasmid p2.1(λU4-6) was used as a template to assay for chicken U4 RNA expression in *X. laevis* oocytes, and pBR322 was used in parallel as a negative control. To perform separate expression studies on the two distinct U4 gene homologies, the 575-bp and 698-bp *Sst*I fragments from p2.1(λU4-6) containing the U4X gene and U4B gene, respectively, were separately cloned into the *Sst*I site of pUC19. Clones were selected in which the orientation of the U4X and U4B genes was in the same transcriptional direction in the pUC plasmid. The clones were designated pU4X(Sst) and pU4B(Sst), respectively. For experiments with these clones, pUC19 was injected in parallel as the negative control. In all injections, an *X. laevis* somatic 5S RNA gene (pXbsF1 [16]) was coinjected as a positive internal control.

Microinjection of DNA and extraction and analysis of RNA were done essentially as described by Yuo et al. (56). Each oocyte was injected with 30 nl of buffer containing 88 mM NaCl, 10 mM Trischloride (pH 7.5), 400 ng of U4 gene (or control) plasmid per μl, 2 ng of 5S gene plasmid per μl, and 8 μCi of [α-^{32}P]GTP (410 Ci/mmol) per μl. For experiments to examine the α-amanitin sensitivity of U4 RNA synthesis, the injection mixtures contained, in addition to the above components, α-amanitin at a concentration of either 1, 10, or 200 μg/ml. Injected oocytes were incubated in modified Barth saline (17) at 19°C for 18 to 20 h. Surviving oocytes were homogenized in 20 μl of homogenization buffer (0.3 M NaCl, 50 mM Trischloride [pH 7.5], 1 mM EDTA, 2% SDS, 1 mg of proteinase K per ml) per oocyte and incubated at room temperature for 30 to 45 min. RNA was extracted once with phenol and once with phenol-chloroform (1:1) and precipitated twice with ethanol (1). The pellet was dissolved in loading dye (99% formamide, 20 mM EDTA), and a sample was run on a 40-cm-long 8% polyacrylamide gel containing 7 M urea. Size markers, consisting of total RNA isolated from chicken liver nuclei, were run in adjacent lanes of the gel. After electrophoresis, the lanes containing the markers were excised and stained with ethidium bromide. The remainder of the gel was subjected to autoradiography.

Genomic DNA Southern blots. With the protocol described in Maniatis et al. (28), genomic DNA was isolated from the liver of four different chickens: a Leghorn rooster, a Leghorn hen, a Cornish hen, and a Barred Rock hen. Six different restriction endonucleases were used in separate digestions of genomic DNA. The DNA fragments generated were separated by electrophoresis in 0.9% agarose gels (approximately 15 to 20 μg of genomic DNA per gel lane) and transferred to nylon filters by a modification of the Southern procedure (49). The DNA was crosslinked to the nylon filters by irradiation for 2 to 3 min on a standard UV transilluminator.

To obtain a hybridization probe highly specific for U4 sequences, a 192-bp *Alu*I-*Rsa*I fragment containing primarily U4B coding sequences was cloned into the *Hinc*II site of pUC18. This fragment (extending from positions 1143 to 1334 in Fig. 2) contains 139 bp of U4B coding sequences and 53 bp of 3′-flanking DNA. This cloned fragment was reisolated from the vector by digestion with *Pvu*II to release a 514-bp fragment containing the 192-bp *Alu*I-*Rsa*I fragment together with adjacent vector DNA. This 514-bp *Pvu*II fragment was denatured and labeled with [α-^{32}P]dCTP and [α-^{32}P]dTTP by using the Klenow fragment of DNA polymerase I and random DNA hexamers (Pharmacia) as primers (14). Specific activities of 2×10^9 Cerenkov cpm/μg of DNA were obtained.

Following prehybridization for 4 to 6 h at 65°C in a solution (6) containing 1% bovine serum albumin, 1 mM EDTA, 7% SDS, 0.5 M Na_2HPO_4, and 0.5 M NaH_2PO_4 (pH 7.0), the filters were hybridized for 12 to 14 h in the same solution containing approximately 1.4×10^8 cpm of labeled probe. The filters were washed twice at 65°C in a solution of 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0])–0.5% SDS for 20 min per wash and twice at 65°C in a solution of 0.1× SSC–0.5% SDS for 20 min per wash. They were then exposed to X-ray film with an intensifying screen at –80°C for 2 to 5 days.

To determine the number of U4 RNA genes in the chicken genome, DNA from the Leghorn hen was used in a genomic reconstruction experiment. For use as an internal standard, the 2.1-kb *Bam*HI fragment was isolated from plasmid p2.1(λU4-6) by two rounds of agarose gel electrophoresis. Based on a chicken haploid genome size of 1.26 pg of DNA (34), it was calculated that 38 pg would represent a single-copy equivalent of a 2.1-kb fragment in 20 μg of genomic DNA. This amount and multiples of it were loaded along with 20 μg of *Apa*I-digested Leghorn hen liver DNA into separate lanes of a gel for Southern analysis. The Southern blot was hybridized with the radiolabeled U4 gene-specific probe described above.

RESULTS

Structure of a region of the chicken genome containing homologies to U4 RNA. A chicken genomic DNA library was screened with a cloned human U4 pseudogene as the hybridization probe. Three distinct overlapping phage clones spanning 25 kb of the chicken genome were subsequently isolated. Restriction maps of these clones are displayed in the

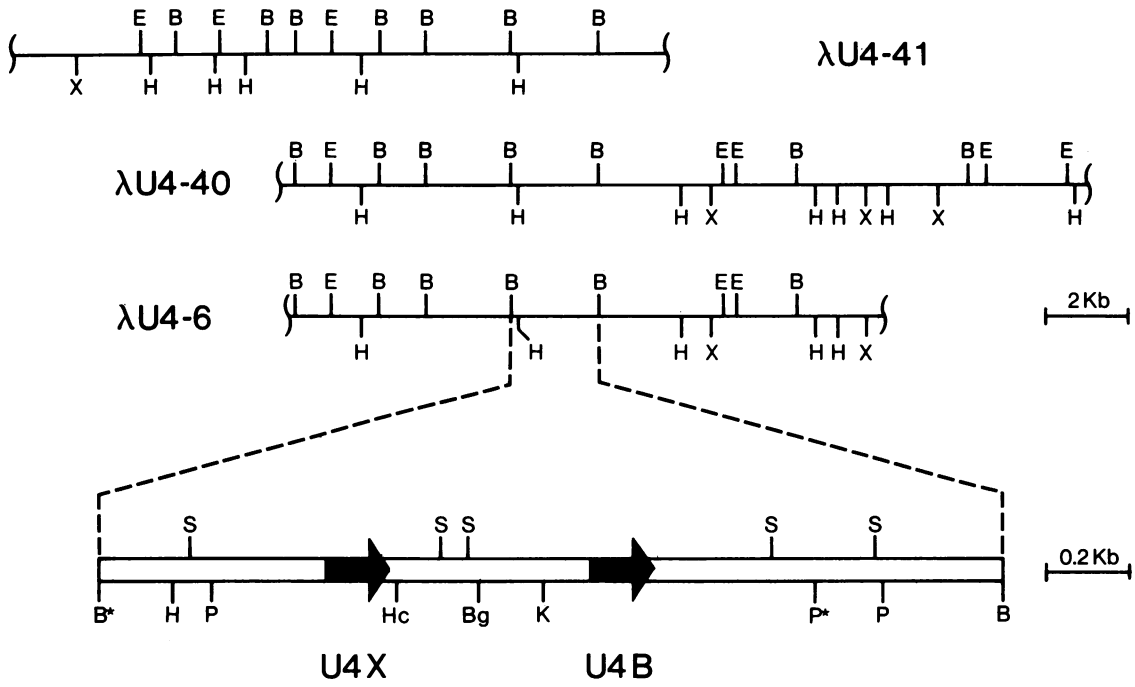

FIG. 1. Chicken genomic DNA clones containing U4 RNA genes. Three phage clones designated λU4-41, λU4-40, and λU4-6 are shown in the upper part of the figure. Wavy lines at the ends of each clone represent *Eco*RI sites in the polylinker of the λ phage vector EMBL-4. A more detailed map of the subcloned 2.1-kb *Bam*HI fragment is shown below the phage maps. The *Bam*HI and *Pst*I sites with asterisks indicate the extent of the region sequenced and reported in Fig. 2. The U4 RNA coding regions are illustrated by bold arrows pointing in the direction of transcription. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sst*I; X, *Xho*I.

upper section of Fig. 1. Southern analysis (not shown) revealed that a 2.1-kb *Bam*HI fragment in each clone contained the sequences hybridizing to the U4 probe. The 2.1-kb *Bam*HI fragment from λU4-6 was subcloned into pBR322 to produce plasmid p2.1(λU4-6). Finer restriction mapping and Southern analysis of this subclone (not shown) revealed that the U4 probe hybridized to two separate subregions within the 2.1-kb fragment.

The lower section of Fig. 1 shows a restriction map of the 2.1-kb fragment, including the locations of the two U4 homologies as determined by DNA sequencing. The upstream homology is designated U4X, and the downstream homology is designated U4B. The sequence of a 1,665-bp region encompassing the two U4 genes is displayed in Fig. 2. The two regions homologous to U4 RNA are shown in larger letters.

Comparison of chicken U4 RNA and cloned U4 DNA sequences. RNA sequencing studies have revealed that two distinct U4 RNA sequence variants exist in higher vertebrates (21, 24, 42). We shall refer to these two RNA sequence variants as U4A and U4B, according to the nomenclature of Reddy (41). Besides having different 3′ termini, the U4A and U4B sequences appear to differ at two internal positions (nucleotides 88 and 99) (41).

The DNA sequences of the two cloned genes from p2.1(λU4-6) were compared with the two published chicken U4 RNA sequences (24, 41) (Fig. 3). As shown, the U4B gene sequence was completely colinear with chicken U4B RNA. It can therefore be assumed that the U4B gene is a true gene capable of coding for chicken U4B RNA. This was supported by the expression studies described below.

On the other hand, the U4X gene sequence did not code for either U4A or U4B RNA. Although more closely related to the U4B sequence, it contained seven base substitutions relative to the U4B gene. Most of these substitutions were clustered very near the 3′ end of the gene (Fig. 3). Interestingly, the presence of a T residue at position 38 is in agreement with the sequences of the human, mouse, rat, and *Drosophila melanogaster* U4 RNAs at that position (37, 41). (Chicken U4A and U4B RNAs seem to be exceptions in having an A at position 38.) Of the six remaining base substitutions relative to U4B, three were identical to bases at the corresponding positions in *Drosophila* U4 RNA, which has a G at position 61, a C at position 129, and a G at position 141 (37). Furthermore, none of the seven base substitutions in the U4X gene would have a significant effect on the proposed secondary structure of the U4 RNA transcript (24) or on the postulated base-pairing interactions between U4 and U6 RNAs in the U4/U6 snRNP particle (4, 19, 43). Because of the specific overall pattern of base substitutions observed, it seems likely that the U4X gene is capable of encoding a functional U4 RNA molecule.

Expression of chicken U4 RNA in oocytes. Initially, the transcriptional potential of subclone p2.1(λU4-6), which contains both the U4X and U4B genes, was tested by microinjection into *X. laevis* oocytes. A 5S RNA gene was coinjected to normalize for the efficiency of injection into the oocyte nucleus (1). Figure 4A (left lane) shows that p2.1(λU4-6) directed the synthesis of a transcript the size of chicken U4 RNA. Injection of pBR322 produced no such transcript (Fig. 4A, right lane).

To determine whether both U4 homologies were being transcribed, U4X and U4B were individually cloned into pUC19 and assayed by separate injections into *X. laevis*

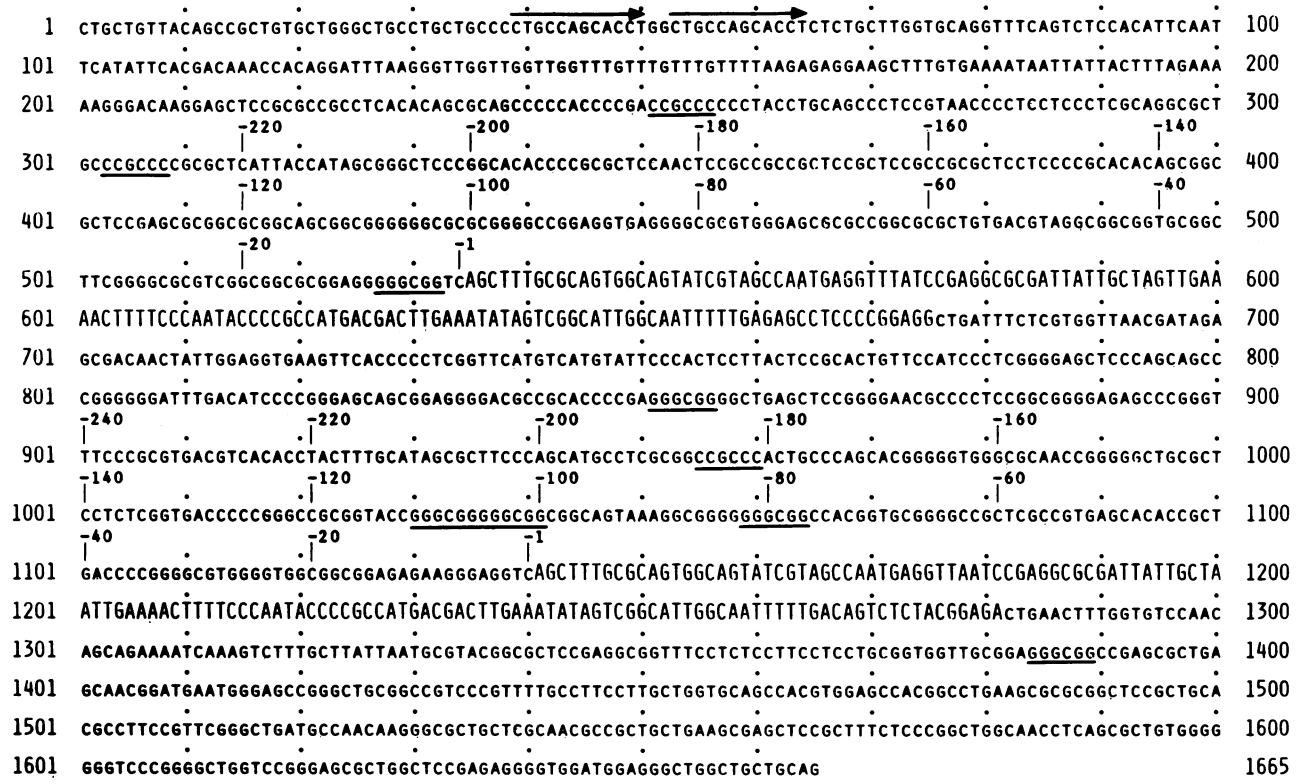

FIG. 2. Nucleotide sequence of a region of the chicken genome containing two genes homologous to U4 RNA. The nontemplate strand of the DNA is shown extending between the *Bam*HI and *Pst*I sites denoted by asterisks in Fig. 1. The two regions homologous to U4 RNA are shown as larger capital letters. The upstream homology (nucleotides 535 through 675) represents the U4X gene, and the downstream homology (nucleotides 1141 through 1281) is the U4B gene. To provide additional points of reference, nucleotides of the 5′-flanking DNA of each gene are designated by negative numbers. Underlining points out the occurrence of the sequence GGGCGG or its complement, which are potential recognition sites for eucaryotic transcription factor SP1 (9). This hexanucleotide is also present at multiple sites upstream of chicken U1 and U2 RNA genes (21a). The horizontal arrows indicate two identical 12-bp direct repeats. It is not known whether these direct repeats have a functional significance.

oocytes (Fig. 4B). A cloned fragment containing the U4B gene, 280 bp of 5′-flanking DNA, and 283 bp of 3′-flanking DNA was capable of directing the synthesis of U4 RNA (Fig. 4B, right lane). On the other hand, no major band the size of mature U4 RNA was observed when the U4X gene with 324 bp of 5′-flanking DNA and 116 bp of 3′-flanking DNA was injected as the template (Fig. 4B, left lane). Instead, compared with the lanes containing the products of pUC19 or U4B gene injection, a darker background of heterogeneous transcripts was observed. The precise origin of these heterogeneous transcripts is unknown at present.

Considerable data have been published showing that the synthesis of U1 and U2 RNAs is inhibited by low concentrations of α-amanitin (11, 32, 36, 44), which is expected for transcripts synthesized by RNA polymerase II. To examine the α-amanitin sensitivity of U4 RNA gene expression, the experiment shown in Fig. 5 was performed. The plasmid containing the U4B gene was injected into oocytes together with increasing amounts of α-amanitin. Figure 5 shows that 1 μg of α-amanitin per ml completely inhibited the synthesis of U4 RNA. In contrast, this amount of α-amanitin had no detectable effect on the synthesis of 5S RNA, which was significantly inhibited only by 200 μg of α-amanitin per ml. Thus, U4 RNA, like the U1 and U2 RNAs, appears to be synthesized by RNA polymerase II.

Genomic organization of chicken U4 RNA genes. To investigate the genomic organization of chicken U4 RNA genes, Southern blots were performed with genomic DNA isolated from four individual chickens (three different breeds). Each of the DNA samples was digested separately with six different restriction enzymes, and the Southern blots were hybridized with a probe highly specific for U4 RNA coding sequences as described in the Materials and Methods.

In all of the DNA samples, the enzymes *Eco*RI, *Pst*I, *Apa*I, and *Pvu*II each generated a single fragment that hybridized to the U4 probe (Fig. 6). Since it is known that none of these four enzymes cuts between the U4X and U4B genes, the single fragment observed in each case would be expected to contain both the U4X and U4B genes. The enzyme *Kpn*I generated two fragments that hybridized to the U4 probe. This was expected, since *Kpn*I recognizes a site between the U4X and U4B genes. Importantly, when DNA from the phage clone λU4-6 was digested with the same enzymes and subjected to Southern analysis (data not shown), bands were observed that were identical in size to those obtained from digestion of genomic DNA. Thus, the phage clones appear to faithfully represent the organization of U4 RNA genes present in chicken genomic DNA.

In the analysis of the genomic DNA samples, *Bam*HI was the only enzyme which gave a result that was partly unpredicted from the phage clones. When digested with this enzyme, DNA from the Cornish and Barred Rock hens

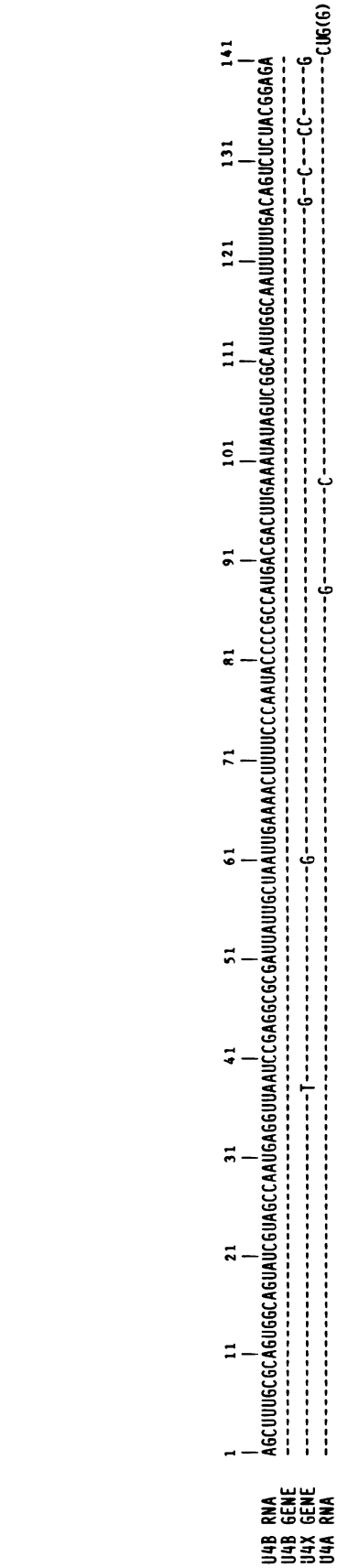

FIG. 3. Comparison of the cloned U4 gene sequences with published chicken U4 RNA sequences. The top line shows the sequence of chicken U4B RNA, except that posttranscriptional base modifications are not indicated (see reference 41 for modified bases). In the lines below, dashes indicate agreement with the U4B RNA sequence, and base changes relative to U4B are shown explicitly.

yielded the predicted 2.1-kb fragment, whereas DNA from the two Leghorns yielded a 6.6-kb fragment in addition to the expected 2.1-kb fragment. However, it is doubtful that this larger *Bam*HI fragment corresponds to a distinct U4 gene locus because none of the other five enzymes used in the analysis provided any evidence for an additional locus. Moreover, the large *Bam*HI band was not present in the DNA from the Cornish and Barred Rock hens. The most likely explanation for the origin of the 6.6-kb band is that it represents an allelic polymorphism. Indeed, a polymorphism resulting in the loss of the *Bam*HI site downstream of the U4B gene would link together the 2.1-kb and the adjacent 4.5-kb *Bam*HI fragments to generate a 6.6-kb fragment. If this interpretation is correct, the two Leghorns must have been heterozygous for the polymorphism, whereas the Cornish and Barred Rock hens were homozygous for the 2.1-kb-fragment.

The results obtained above indicate that all of the U4 sequence homologies in the chicken genome must be closely linked or present in essentially the same genomic sequence environment. To determine the number of copies of the U4X and U4B loci in the chicken genome, the genomic reconstruction experiment shown in Fig. 7 was performed. DNA

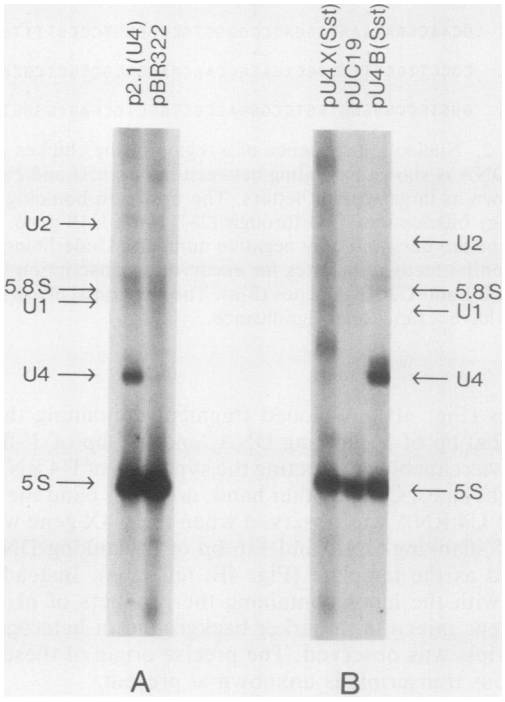

FIG. 4. Transcription of cloned chicken U4 RNA genes in oocytes. Nuclei of frog oocytes were injected with plasmids containing chicken U4 RNA genes (or with vector plasmids only), together with [α-^{32}P]GTP. A plasmid containing an *X. laevis* 5S RNA gene was coinjected in each case to normalize for efficiency of injection into the oocyte nucleus (1). Samples of extracted RNA were electrophoresed on an acrylamide gel and autoradiographed. A second gel was then run, normalizing each lane to include an equivalent amount of 5S RNA transcript. The results from the normalized gel are shown. The arrows point out the distances migrated by chicken U2, 5.8S, U1, U4, and 5S RNA species run in adjacent lanes as size markers. (A) RNA from oocytes injected with p2.1(λU4-6), which contains both the U4X and U4B genes (left lane) and RNA from oocytes injected with pBR322 (right lane). (B) RNA from oocytes injected with plasmids containing the U4X gene (left lane); the U4B gene (right lane); and pUC19 (center lane).

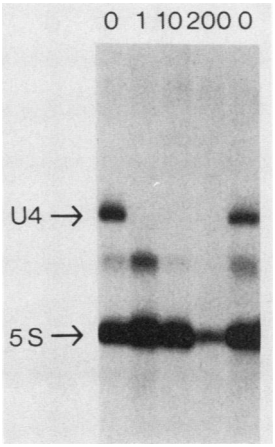

FIG. 5. Sensitivity of U4 RNA synthesis to α-amanitin. The U4B gene (cloned into the pUC19 vector) was injected into oocytes together with 0, 1, 10, or 200 μg of α-amanitin per ml as shown at the top of each lane. A plasmid containing an *X. laevis* 5S RNA gene was coinjected in each case as an internal control. RNA extracted from the equivalent of five oocytes was loaded into each lane. The results indicate that U4 RNA synthesis is completely inhibited by 1 μg of α-amanitin per ml whereas 5S RNA synthesis is significantly inhibited only at the 200 μg/ml concentration of α-amanitin. (The light band that runs between the 5S and U4 RNA bands is an RNA polymerase III transcript apparently arising from sequences in the 3′-flanking DNA of the U4B gene [unpublished data].)

from the Leghorn hen was digested with *Apa*I, and samples were loaded into four separate lanes of an agarose gel for Southern blotting. (*Apa*I digestion generated a single 3.1-kb band that contained both the U4X and U4B genes [Fig. 6].) The purified 2.1 -kb *Bam*HI fragment that contains the U4X and U4B genes was included as an internal standard in each lane of the gel in an amount equivalent to either 0, 1, 3, or 9 copies per haploid chicken genome. The resultant Southern blot was hybridized with the U4 sequence probe described above. The relative intensity of the 3.1-kb band in Fig. 7 indicates that the U4X and U4B genes are each probably present as a single copy in the chicken genome.

All of the data obtained on the organization of U4 RNA genes are consistent with there being only two U4 sequence homologies per haploid genome in the four chickens examined (and in the genome of the chicken from which the λ phage library was constructed). However, the results are somewhat unexpected in that we did not detect a separate gene to code for the published chicken U4A RNA sequence (24, 41). We believe it is very unlikely that a third gene is closely linked to the U4X/U4B locus and has escaped detection. For example, the enzyme *Pst*I, which cuts 267 bp 5′ of the U4X gene and also 383 bp 3′ of the U4B gene, generated only a single band in genomic DNA Southern blots (Fig. 6). Moreover, extensive Southern analysis of the phage clones with numerous additional enzymes failed to reveal any evidence for a third U4 homology in the phage clones (data not shown). One possible explanation for the apparent lack of the distinct U4A gene is that it may be an allele of either the U4X gene or the U4B gene. In that case, the U4A gene would not be detectable as a distinct gene in the genomic Southern blots. In this regard, it is interesting that the first three nucleotides in the 3′-flanking DNA of both the U4X and U4B genes (CTG at positions 676 to 678 and 1282 to 1284 in Fig. 2) are in agreement with the nucleotides at the 3′ terminus of U4A RNA (CUG), which is three (or four) nucleotides longer than U4B RNA (Fig. 3) (24, 41).

It is known that the U4 and U6 RNAs are associated with each other in vivo in the same snRNP particle (4, 19). In

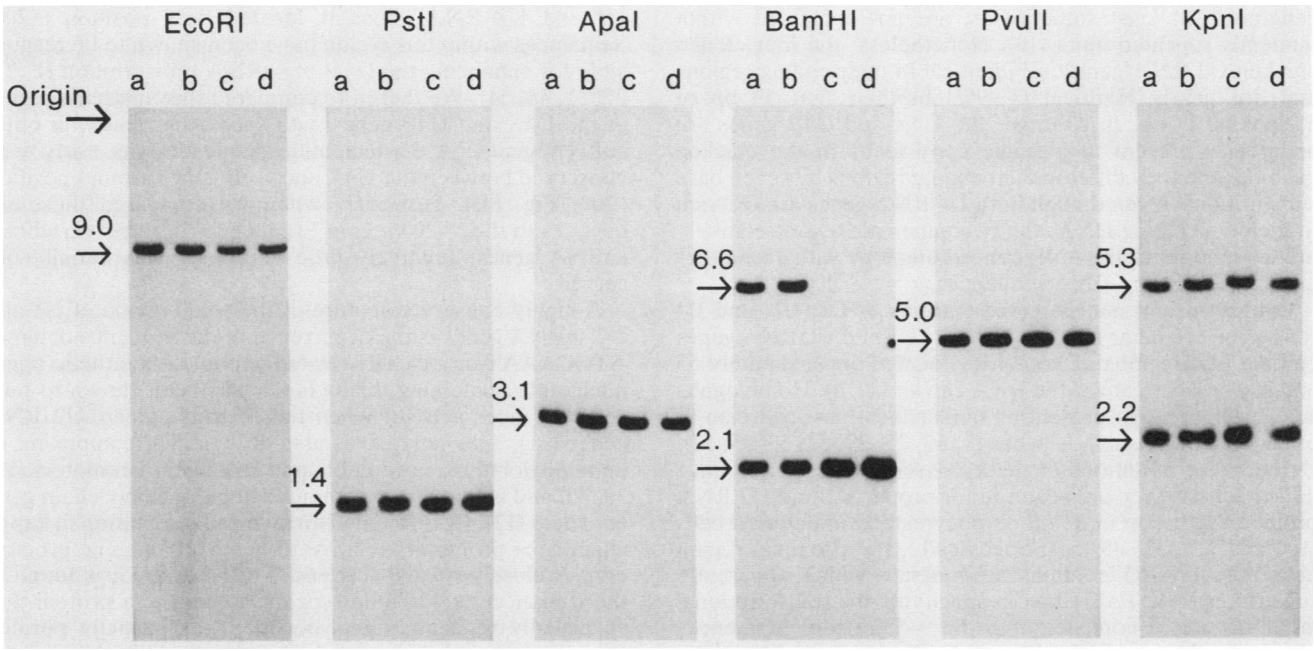

FIG. 6. Genomic organization of chicken U4 RNA genes. Liver DNA from four individual chickens was digested with six different restriction endonucleases as shown above each panel, electrophoresed on a 0.9% agarose gel, transferred to nylon filters, and hybridized with a probe specific for U4 sequences. The lanes contained DNA from (a) Leghorn rooster, (b) Leghorn hen, (c) Cornish hen, and (d) Barred Rock hen. The figure is a composite of autoradiograms taken from a number of different Southern gels in which the samples did not necessarily migrate the same distance. The numbers on the left of each panel designate the size (in kilobase pairs) of the hybridizing fragments, based on the sizes of markers run in the individual gels.

Saccharomyces cerevisiae, a single snRNA molecule contains primary sequence homology to both the U4 and U6 RNAs of higher eucaryotes (52). Therefore, we performed experiments to determine whether U6 RNA genes might be closely linked to the cloned chicken U4 RNA genes. Using an oligonucleotide probe complementary to U6 RNA, we were unable to detect any U6 RNA homologies in the region of the chicken genome represented by the phage clones shown in Fig. 1 (data not shown). Hence, there is apparently no close linkage of U6 RNA genes with the U4X/U4B locus in the chicken genome. A recent study has indicated that U4 and U6 RNA genes are also not closely linked in the *Drosophila* genome (45).

DISCUSSION

We have isolated from a chicken DNA library three nonidentical but overlapping phage clones that hybridize to a U4 RNA sequence probe. These three clones together extend over 25 kb of the chicken genome and represent a single genetic locus. The two U4 homologies contained in these clones are closely linked within 465 bp of each other and have been designated the U4X and U4B genes. The cloned U4B gene is almost certainly a true U4 gene since it is colinear in sequence with chicken U4B RNA and is expressed after injection into frog oocytes. The transcriptional potential of the U4X gene is discussed further below.

Comparison of U1, U2, and U4 RNA gene organization in the chicken. Although the U1, U2, and U4 RNAs are found in similar snRNP particles and probably carry out related metabolic functions, the genes that encode these RNAs are organized differently from each other in the chicken genome. For example, the chicken U2 RNA genes are present in 35 to 40 copies per haploid chicken genome, and each copy occurs within an identical tandemly repeated unit 5.35 kb long (21a). The chicken U1 RNA genes, although clustered in the genome in at least some cases, are not contained within tandemly repeated units (10). Nonetheless, the four cloned chicken U1 RNA genes are identical in their coding regions and are nearly identical (~99%) in their first 59 bp of 5′-flanking DNA. In contrast, the U4X and U4B genes are apparently present at a single copy locus in the chicken genome, and they differ in their coding regions by seven base substitutions. Even though both U4 RNA genes are GC-rich in their 5′-flanking DNA, the two genes possess a relatively limited amount of DNA sequence homology with each other in their 5′-flanking DNA sequences.

Conserved and nonconserved features of U1, U2, and U4 RNA gene promoter regions. Chicken U1 and U2 RNA genes contain two regions of homology located approximately 55 and 200 bp upstream of the RNA cap site (21a). Homologous sequences are also present at similar locations upstream of other vertebrate snRNA genes (1, 10, 25, 29, 51, 53–55, 57). Deletions or mutations of these conserved sequences have indicated that they are required for proper U1 and U2 RNA promoter function in *X. laevis* oocytes and in cultured cells (1, 7, 25, 27, 31, 48, 54). Specifically, the proximal region near position –55 contains a sequence which apparently substitutes for a TATA box in specifying the transcriptional initiation site. A comparison of the "–55 region" sequences of seven cloned chicken snRNA genes showed that the sequence CGCCGTG was highly conserved at nearly identical positions upstream of each of the chicken snRNA gene cap sites (the underlined nucleotides are conserved in all seven genes) (Fig. 8A). Similar sequences, particularly the pentanucleotide CCGTG (or close variants of it), are present

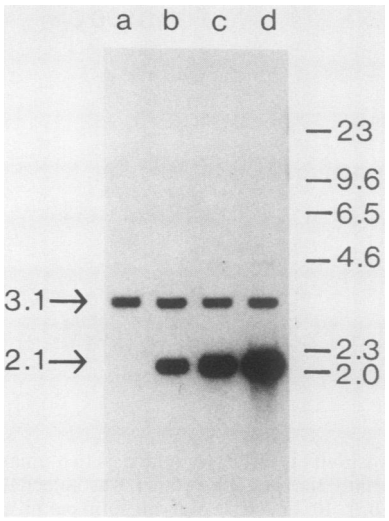

FIG. 7. Copy number of U4 RNA genes in the chicken genome. Genomic DNA from the Leghorn hen was digested with *Apa*I, electrophoresed on an agarose gel, and blotted to a nylon filter. Each lane of the gel also contained the equivalent (per haploid genome) of either 0 (lane a), 1 (lane b), 3 (lane c), or 9 (lane d) copies of the unlabeled 2.1-kb *Bam*HI fragment as an internal standard. The Southern blot was hybridized with a probe specific for U4 sequences. The band at 3.1 kb represents hybridization of the probe to the U4X and U4B genomic sequences. The intensity of the 3.1-kb band relative to that of the internal standards (at 2.1 kb) indicates that there is approximately one copy of the U4X/U4B locus per haploid chicken genome. Sizes of markers are indicated to the right (in kilobase pairs).

at nearly identical locations upstream of other vertebrate U1 and U2 RNA genes.

A second upstream region that is conserved in vertebrate U1 and U2 RNA genes is located near position –200. Sequences within this region have been shown to be responsible for enhancing the level of snRNA transcription (1, 25, 27, 31, 48, 54). We therefore compared this upstream region of the U4X and U4B genes with each other and with other snRNA genes. A 35-nucleotide sequence was fairly well conserved between the U4X and U4B genes around position –200 (Fig. 8B). However, when we compared these sequences to the "–200 region" sequences of other vertebrate snRNA genes, relatively little homology was initially observed.

A highly conserved feature of the –200 region of U1 and U2 snRNA genes is the occurrence of the sequence octamer ATGCAAAT or a closely related variant. A synthetic oligonucleotide containing this octamer has been shown to possess enhancer activity when linked to *X. laevis* U2 RNA genes (31). This octamer is also an essential component of immunoglobulin heavy and kappa chain gene promoters (13, 30, 40) and is found in the immunoglobulin heavy chain gene enhancer (12). (It is found in an inverted orientation in kappa chain gene promoters relative to heavy chain gene promoters.) A closely related sequence, ATGCAAAG, is found in the simian virus 40 72-bp repeats. Recently, a protein that specifically recognizes this octamer was partially purified from both lymphocytes and HeLa cells (47). Unlike the previously characterized vertebrate U1, U2, and U3 RNA genes, which contain variants of the octamer on the nontemplate strand (1, 25, 27, 31), the cloned U4 RNA genes contain variants of the sequence on the template strand. In the –200 region the U4B and U4X genes contain the se-

A

CHICKEN U4X	-63	G	C	G	C	G	C	T	G	T	G	A	C	G -51
CHICKEN U4B	-61	G	C	T	C	G	C	C	G	T	G	A	G	C -49
CHICKEN U2	-63	C	G	C	G	T	C	C	G	T	G	A	G	C -51
CHICKEN U1 52A	-62	G	G	T	C	G	C	C	G	T	G	C	G	G -50
CHICKEN U1 52B	-62	G	G	T	C	G	C	C	G	T	G	C	G	G -50
CHICKEN U1 52C	-62	A	G	T	C	G	C	C	G	T	G	C	G	G -50
CHICKEN U1 2.5	-62	G	A	A	C	G	C	C	G	T	G	C	G	G -50
G		5	4	1	1	6	0	0	7	0	7	0	6	5
A		1	1	1	0	0	0	0	0	0	3	0	0	
T		0	0	4	0	1	0	1	0	7	0	0	0	
C		1	2	1	6	0	7	6	0	0	0	4	1	2
CHICKEN SNRNA "-55" CONSENSUS		G	G	T	C	G	C	C	G	T	G	C	G	G

B

CHICKEN U4B	-221	CTACTTTGCATAGCGC-TTCCCAGCATGCCTCGCG	-188
		** ** ******* +*****+*****+*****+*****	
CHICKEN U4X	-222	CTCATTACCATAGCGGGCTCCCGGCACACCCCGCG	-188

C

CHICKEN U4B (TEMPLATE)	-208	GCTATGCAAAGTA-GGTGTGACG			-229
FROG U2 (NON-TEMPLATE)	-272	GCTATGCAAA-TAGGGTGTGCCGGG			-249
CHICKEN U4X (TEMPLATE)	-191	GGGGTGTGCCGGG.....ATGGTAAT			-219
RAT U1 (NON-TEMPLATE)	-222	ATGCAGAT.....AGGGTCTGCCGGG			-191
MOUSE U6 (TEMPLATE)	-221	ATGCAAAT.....AGGGAGTGCCGAG			-251

FIG. 8. Sequence homologies in the promoter regions of chicken and other vertebrate snRNA genes. (A) Comparison of the −55 region squences of seven cloned chicken snRNA genes. In the consensus sequence shown, larger capital letters indicate bases conserved in at least six of the seven gene sequences. Underlined nucleotides are perfectly conserved in all seven gene sequences. References: chicken U1 (10); chicken U2 (21a). (B) Comparison of sequences in the –200 region of the U4B and U4X genes. Asterisk indicates conserved bases; + indicates pyrimidine/pyrimidine or purine/purine conservation. The underlined sequences are homologous to the consensus octamer ATGCAAAT when read on the opposite strand in a 5′ to 3′ direction. (C) Comparison of sequences in the −200 region of various vertebrate snRNA genes. The underlined regions are homologous to the ATGCAAAT octamer. The sequences are aligned around a second conserved sequence found in the vicinity of the octamer. Dots indicate nucleotide positions not important to the present discussion. In comparison to the genes shown, other cloned vertebrate snRNA genes exhibit less homology to the U4 sequences. References: frog U2 (32); rat U1 (53); mouse U6 (38).

quence variants ATGCAAAG and ATGGTAAT, respectively, reading 5′ to 3′ on the template strand (underlined region in Fig. 8B). Hence, in the U4 RNA genes the octamer appears to be inverted relative to its orientation in the U1, U2, and U3 RNA genes of chickens and other vertebrates.

Having made this observation, we looked for homologies between template strands of the U4 RNA genes and nontemplate strands of other vertebrate snRNA genes. The best homology found in the −200 region was between the chicken U4B RNA gene and an *X. laevis* U2 RNA gene (Fig. 8C). As shown, 20 of 23 nucleotide positions were conserved between these two genes, but on opposite strands. In addition to the ATGCAAAT consensus octamer, an adjacent sequence, GGTGTG(A/C)CG, was also well conserved. Notably, sequences similar to this homology are found in the template strand of the U4X gene and in the nontemplate strand of a rat U1 RNA gene (Fig. 8C), but at different positions relative to the conserved octamer. A mouse U6 RNA gene (38) contains both conserved elements, like the U4 RNA genes, on the template DNA strand. Thus, some sequence elements in the −200 region of the U4 and U6 RNA genes have an inverted orientation relative to homologous sequences upstream of U1 and U2 RNA genes. Perhaps these represent examples of the natural occurrence of enhancer sequence inversions during evolution. Additional features that may play a role in chicken U4 RNA gene expression are pointed out in Fig. 2.

Is the U4X gene a functional gene? If transcribed, the U4X

gene codes for a U4 RNA sequence variant not previously described for chicken cells. Although we have no direct evidence that it is transcriptionally active, several lines of evidence based on nucleotide sequence data suggest that it may be a functional gene. First, the pattern of base substitutions in the coding region of the gene is such that the product is very likely a functional U4 RNA. Second, the U4X and U4B genes have similar sequences in their 5'-flanking DNAs at locations that are known to be involved in snRNA gene expression. Third, in the 3'-flanking DNA, both genes contain a sequence that is a reasonably good match to a conserved sequence (consensus $GTTTN_{0-3}AAAPuN$-NAGA) that is involved in the formation of the 3' ends of snRNAs (20, 56). The U4X and U4B genes contain the sequences $GGTTN_0AACGNNAGA$ and $GTGTN_2AACAN$-NAGA, respectively.

In any case, the failure of the U4X gene to be efficiently expressed in the heterologous oocyte system should not be taken as conclusive evidence that it is a nonexpressible pseudogene. Perhaps the U4X gene is expressed constitutively at a low level in chicken cells, or more interestingly it may be expressed in a tissue-specific or development stage-specific manner. There is precedence for the developmental regulation of snRNA gene expression in the case of the mouse (26) and frog (15) U1 RNA genes. If expression of the U4X gene is regulated, a gene-specific factor, not present in oocytes, may be required for expression from the U4X gene promoter.

In conclusion, the U4 RNA genes reported here share certain features found in the promoter regions of previously characterized snRNA genes of chickens and other vertebrates. However, some aspects of the U4 promoter structure are different and may be unique to U4 RNA gene expression. The transcription of snRNA genes is undoubtedly dependent on the utilization of common transcription factors (25, 31, 54), some of which are very likely shared with other RNA polymerase II promoters as well. It will therefore be of interest to determine whether additional class-specific and gene-specific factors might also be involved in snRNA gene expression.

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