Functional elements of the ribosomal protein L7a (rpL7a) gene promoter region and their conservation between mammals and birds

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ABSTRACT
The transcriptional initiation sites of the chicken ribosomal protein L7a (rpL7a) gene have been determined and found to occur at three consecutive cytidine residues at the start of a polypyrimidine tract of 8 base pairs (bp). A comparative analysis of the 5’ upstream regions of the mouse, human and chicken rpL7a genes identified two sequence elements (Box A and Box B) conserved over the 600 million years of divergent evolution that separate mammals and birds. Only Box A (nts -56 to -39) and Box B (nts -25 to -4) sequences were detected to bind nuclear factors from mouse nuclear extracts in an analysis of the mouse rpL7a 5’ upstream sequence. Box A and Box B bind different nuclear factors and the factor binding to mouse Box A and mouse Box B sequences could be effectively competed by corresponding homologous sequences from the human and chicken rpL7a promoters. These results indicate that elements of the rpL7a promoter region are conserved between mammals and birds. An in vivo analysis of the mouse rpL7a 5’ upstream sequence required for efficient transcription identified the 5’ border of the minimal promoter region as lying between nts -50 and -56. Constructs containing 56 bp of 5’ upstream DNA and the first 25 bp rpL7a exon were very efficiently transcribed indicating that sequences within the first intron are not required for gene expression. No sequence similarity was detected between the rpL7a promoter elements and described promoter elements of other eukaryotic ribosomal protein genes.

INTRODUCTION
The biosynthesis of mammalian ribosomes involves the expression of four RNA molecules and 70–90 structurally diverse and evolutionarily unrelated ribosomal proteins (rps) whose expression is coordinately regulated during growth and development (1, 2). In murine cells, it has been shown that the stoichiometric balance of the various ribosomal proteins are a result of their respective mRNAs being transcribed and translated with similar strengths and efficiencies (3). Control of expression of the ribosomal proteins can take place both at the transcriptional level and the post-transcription level. The promoters of only five mammalian ribosomal protein genes have been analyzed to any detail (4–11). This is, in the mainly results from the difficulty in isolating functional intron-containing ribosomal protein genes in the presence of their multiple processed pseudogenes which are also present in mammalian genomes (12).

The mouse Surfeit locus contains a tight cluster of at least six genes (Surf-1 to -6) whose unusual organization suggests cis-interaction and/or coregulation between the genes (13). The topography of the Surfeit locus is so far unique for a cluster of vertebrate genes and the organization of the locus and the juxtaposition of the genes is conserved in man (14) and bird (15). The Surf-3 gene has been identified as the single functional intron-containing gene encoding the mouse ribosomal protein L7a (rpL7a) (12, 16, 17). The mouse Surf-3/rpL7a gene contains eight exons and seven introns spread over 3 kilobases and is highly transcribed to produce a 1 kb mRNA (17). Both the DNA and amino acid sequence of the rpL7a gene are highly conserved through evolution (15, 16, 18–22).

The 5’ end of the mouse Surf-3/rpL7a gene contains several features common to a number of mammalian ribosomal protein genes including a very small first exons, a very short untranslated leader, transcription start sites at cytidine residues embedded in a polypyrimidine tract, the absence of a canonical TATA box preceding the gene and a location in a CpG-rich island (23). It has been found that the 5’ end and upstream sequence of the human Surf-3/L7a gene is able to activate a truncated TRK oncogene (22, 24). Furthermore rpL7a expression has been found to be induced after U.V. irradiation of xeroderma pigmentosa cells (25) and to be responsive to thyrotropin (TSH) (26). In this...
communication we have determined the 5' upstream sequence of the human and chicken rpL7a genes and analyzed the cis acting sequences and trans acting factors involved in the transcription of the mouse rpL7a gene. We show that some functional elements of the rpL7a promoter region have been conserved over the 600 million years of divergent evolution which separates mammals from birds.

MATERIALS AND METHODS

DNA cloning and analysis

A human cosmid library and a λ charon 28 chicken genomic library (gift of Dr. T. Graf) were screened at low stringency hybridization conditions using a mouse full length cDNA Surf-3/rpL7a probe. Restriction fragments carrying the Surf-3/rpL7a gene were subcloned into Bluescript vectors (Stratagene) and sequenced using T7 DNA polymerase (Sequenase, USB) according to the manufacturer's instructions. Sequence analysis was carried out using the Wisconsin software (27).

Plasmid construction

A 2.3 kb BglII-BamHI fragment containing the 5' end sequence of the mouse Surf-3/L7a gene and 1.2 kb of the 5' flanking region was isolated and cloned in a Bluescript vector (Stratagene). A polymerase chain reaction (PCR) was carried out using two oligonucleotide lying between nt 1 and nt 21 (also containing synthetic SacII and XbaI restriction cleavage sites at the 5' end) and nt 1388 and nt 1415, respectively, amplifying a 1425 bp fragment containing the whole first intron and the first exon of the Surf-3/rpL7a gene and 1.2 kb of upstream sequence. (Δ1 clone). A second clone, without first intron sequences, was generated by PCR using two synthetic oligonucleotides, one containing sequences between nt 1 and nt 21, as described above, and a second oligonucleotide from nt 1205 to nt 1237 and contains the sequence from the first coding methionine to 1200 bp upstream (clone Δ7). Another clone (Δ5*) was generated by PCR with an oligonucleotide (5'-CCTGAGATCGGATATCTGC-3') lying between nt 1157 and nt 1177 bearing a trinucleotide change which destroyed the Box A binding site (see mutant A Fig 3 for details) and an oligo lying between nt 1388 and nt 1415 to study the influence of this element on the expression of Surf-3/rpL7a gene. PCR fragments were fractionated on a 1% agarose gel, purified, kinased and cloned into a vector expressing the neomycin gene. The neomycin gene fused to a thymidine kinase gene. The neomycin gene and determined the DNA sequence of the 5' upstream regions of both the human and chicken rpL7a genes. The genomic structures and cDNA sequences of the mouse, human and chicken Surf-3/rpL7a genes and the transcriptional start sites of the mouse and human rpL7a genes have previously been reported (15, 17, 19, 22, 23). The mouse, human and chicken genes show a high degree of sequence homology within their coding regions but little is known about the regulation of expression of these genes. In order to make a comparative analysis of the rpL7a promoter regions in the two species we first identified the transcriptional start sites of the chicken Surf3/rpL7a gene and determined the DNA sequence of the 5' upstream regions of the human and chicken genes. A primer extension analysis was performed to determine the transcription start sites and the length of the 5' untranslated leader of the chicken rpL7a gene. The results (Fig 1) show that the chicken rpL7a gene contains three start sites, one at each of the three C residues in a polypyrimidine tract of 8 bp.

The 5' upstream regions of both the human and chicken rpL7a genes were isolated from previously cloned genomic clones (15, 19) and sequenced in order to identify elements also present in the mouse rpL7a promoter. The presence of conserved elements in the 5' non coding region of the rpL7a genes of mammals and birds, which are separated by 600 million years of divergent evolution (15), might suggest an important role for these sequences in rpL7a gene expression. The relative positions of

RESULTS

Identification of the transcriptional start sites of the chicken Surf3/rpL7a gene and a comparative analysis of the mouse, human and chicken rpL7a promoter regions

The genomic structures and cDNA sequences of the mouse, human and chicken Surf-3/rpL7a genes and the transcriptional start sites of the mouse and human rpL7a genes have previously been reported (15, 17, 19, 22, 23). The mouse, human and chicken genes show a high degree of sequence homology within their coding regions but little is known about the regulation of expression of these genes. In order to make a comparative analysis of the rpL7a promoter regions in the three species we first identified the transcriptional start sites of the chicken gene and determined the DNA sequence of the 5' upstream regions of the human and chicken genes. A primer extension analysis was performed to determine the transcription start sites and the length of the 5' untranslated leader of the chicken rpL7a gene. The results (Fig 1) show that the chicken rpL7a gene contains three start sites, one at each of the three C residues in a polypyrimidine tract of 8 bp.

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the transcriptional start sites and a comparative analysis of the upstream regions of the three rpL7a genes are shown in Fig. 2. It can be seen that a very high degree of homology exists between the three rpL7a genes between nt -70 and the first ATG suggesting that elements involved in the expression of the Surf-3/rpL7a genes might be found in this small region upstream and around the start sites of transcription.

**Functional characterization of the mouse Surf-3/rpL7a promoter**

To assess the functional in vivo role of the conserved sequences in Box A that are able to bind nuclear factors in vitro, progressive deletions of the 5' end of the mouse rpL7a gene were inserted upstream of a promoterless neomycin gene (Δ1 to Δ7). Expression of the different fusion constructs was assessed after transfection into BALB/c 3T3 cells (Fig. 6). Total cytoplasmic RNA from the transfected cells was assayed by S1 mapping after transient cotransfection with a reference plasmid (PSV2CAT) to provide an internal reference for transfection efficiency. This S1 analysis shows that elements essential for the efficient expression of the rpL7a gene are contained within a fragment which minimally contains 56 bp of 5' untranslated sequence (see clone Δ5 Fig. 6). The maximum homology between the promoter regions of human, chicken and mouse

**Binding sites for nuclear factors in the Surf-3/rpL7a promoter region**

To test the notion that the 5' upstream sequence elements conserved between the three rpL7a gene promoters might bind transcription factors involved in the expression of Surf-3/rpL7a, an extensive group of synthetic oligonucleotide, covering the mouse rpL7a promoter region between nt. -95 to nt. +1 was utilized in gel retardation assays. Only two short oligonucleotides, which both contain sequences conserved between mouse, human and chicken (Fig 2), were found to bind nuclear factors in the in vitro gel retardation assays using a L cell nuclear extract (Figs 3 & 4). One region contained sequences between nt. -56 to nt. -35 (Box A) (Fig. 3). The complex formed by this site is efficiently competed by the same unlabelled oligonucleotide (nt. -56 to nt. -35) but is not competed by an oligonucleotide bearing a three nucleotide substitution from the conserved region (nt. -52 to nt. -50) (see Fig 2 Box A and lane A in Fig 3). Oligonucleotides from corresponding region of human and chicken rpL7a promoters (see Fig 2) are able to effectively compete with the mouse Box A region (see lanes H Box A and C Box A in FIG. 3) strongly suggesting that the nuclear factor(s) that binds to this site are conserved between the three species.

The second conserved rpL7a promoter region found to bind nuclear factors was from nt. -25 to -4 (Box B) (see Fig 2). This sequence was further defined by oligonucleotides containing mutations within this box (Fig 4). One, Mutant A, contained changes at nts. -18 to -20 and the second, Mutant B, contained changes at nts. -12 to -14. Both mutant oligonucleotides were unable to compete with the wild type sequence defining the binding region. In addition, competition experiments with the corresponding wild type human and chicken sequences strongly indicate that the nuclear factors binding the Box B region are also conserved between mammals and birds. Furthermore the absence of competition between the mouse Box A and Box B oligonucleotides demonstrates that the two regions bind distinct nuclear factors (Fig. 5).

**Functional characterization of the mouse Surf-3/rpL7a promoter**

To assess the functional in vivo role of the conserved sequences in Box A that are able to bind nuclear factors in vitro, progressive deletions of the 5' end of the mouse rpL7a gene were inserted upstream of a promoterless neomycin gene (Δ1 to Δ7). Expression of the different fusion constructs was assessed after transfection into BALB/c 3T3 cells (Fig. 6). Total cytoplasmic RNA from the transfected cells was assayed by S1 mapping after transient cotransfection with a reference plasmid (PSV2CAT) expressing the chloramphenicol acetyltransferase (CAT) gene. A 208 bp S1 probe which spans the two mouse rpL7a cap sites (major and minor sites separated by 4 bp (23)) yields a major 191 bp fragment and a minor 195 bp fragment when protected by the rpL7a/neomycin fusion gene mRNA initiated at the correct transcription start sites (see Fig. 6). Balb/c 3T3 cells were transfected with the different fusion genes together with

![Fig 1. Primer extension analysis of the 5' end of the chicken Surf-3/rpL7a gene. The lane on the left (P. EXT) of the gel shows a primer extension carried out with a 15 bp primer (see Materials and Methods). The four tracks on the left contain M13 DNA sequence size markers. At the bottom the three major start sites at C residues are indicated in the chicken rpL7a sequence by arrows.](https://academic.oup.com/nar/article-abstract/20/13/3367/2383398/Functional-elements-of-the-ribosomal-protein-L7a/figure1)

![Fig 2. Comparative analysis of the mouse, human and chicken rpL7a promoters. The 5' upstream and first exon sequence are shown in uppercase letters and first few bp of the first intron are shown in lowercase letters. Nucleotide identities between the mouse and human sequences and human and chicken sequences are indicated by stars. Numbers start from the mouse rpL7a major transcriptional initiation site denoted by +1. The start sites of transcription of each of the three genes are indicated by double underlining. The first methionine codon (ATG) of each gene is underlined. The Box A and Box B elements identified in the in vitro (Figs 3 & 4) and in vivo (Fig 6) analyses are boxed. The 5' endpoints of the position of the deletion clones Δ4, Δ5 and Δ6 used for the in vivo analysis (see Fig 6) are indicated.](https://academic.oup.com/nar/article-abstract/20/13/3367/2383398/Functional-elements-of-the-ribosomal-protein-L7a/figure2)
rpL7a genes are present in this 56 bp and -56 is the border of Box A which contains sequences able to bind nuclear factors in vitro. (see Figs 2 & 3). A mutant Δ5 fusion construct, Δ5*, containing mutations at nt -50 to -52 which destroy Box A binding activity in vitro (see mutant A in Fig 3) was greatly inhibited in the level of RNA expression (Fig 6). This shows that sequences within Box A are required for both in vitro binding to nuclear factors and efficient expression in vivo and that the border of the minimal 5' upstream sequence needed for efficient rpL7a promoter activity lies between nt -56 and -50.

With some mammalian ribosomal protein genes, sequences within the first intron appear to play a role in the control of transcription (33). No major differences could be detected between the expression of a construct containing the first intron (Δ1) and a construct in which the first intron was deleted (Δ7) (Fig 6) indicating that in the case of the rpL7a gene, DNA sequences within the first intron are not required for efficient transcription.

**DISCUSSION**

The transcription initiation sites of the chicken rpL7a gene and the DNA sequences of the 5' upstream regions of the human and chicken rpL7a genes have been determined and compared to similar sequences in the mouse rpL7a gene in order to identify conserved elements that may be involved in rpL7a gene expression. The chicken rpL7a gene has a number of characteristics in common with mammalian rpL7a genes and other mammalian rp genes including a short 5' untranslated leader sequence, a short first exon, relatively small introns, and transcription initiation sites at C residues embedded in a polypurine tract. There are three different start sites for the chicken rpL7a gene at consecutive C residues in the polypurine tract. Transcription initiation of ribosomal protein genes at C residue embedded in a polypurine tract is found

Fig. 3. Gel retardation analysis of the Box A site. A labelled wild type mouse oligonucleotide from nts -56 to -39 encompassing the Box A site (see sequence beneath gel and Fig 2) was incubated in the absence (-) or presence (+) of mouse L cell nuclear extract. The third to fifth lanes of the gel show the effect of competition of the labelled oligonucleotide with increased amounts of cold wild type oligonucleotide (1, 10, 50 ng). Lane A shows a competition with a 50 ng cold oligonucleotide mutated at nucleotides -50 to -52 (see Mutant A sequence beneath gel). Lane H Box A and C Box A show competitions with mouse wild type Box A labelled oligonucleotide with 50 ng cold human and chicken corresponding sequence (see H Box A and C Box A sequences beneath gel and Fig 2). The position of protein-DNA complex and the free oligonucleotide DNA are indicated.

Fig. 4. Gel retardation analysis of the Box B site. A labelled mouse wild type oligonucleotide from nts -25 to -4 encompassing the Box B site (see sequences beneath gel and Fig 2) was incubated in the absence (-) or presence (+) of mouse L cell nuclear extract. The third to fifth lanes of the gel show the effect of competition of the labelled oligonucleotide with increased amounts of cold wild type oligonucleotide (1, 10, 50 ng). Lanes A and B show competitions with 50 ng cold oligonucleotides mutated at nucleotides -18 to -20 and nucleotides -12 to -14 respectively (see Mutant A and B sequences beneath gel). Lanes H Box B and C Box B show competitions with mouse wild type Box B labelled oligonucleotide with 50 ng cold human and chicken corresponding sequence (see H Box B and C Box B sequences beneath gel and Fig 2). The position of protein-DNA complex and the free oligonucleotide DNA are indicated.
in all vertebrate species so far analyzed including Drosophila (34, 35), Xenopus (36-40) and mammals (3, 23, 41-46). The polypyrimidine tract is thought to affect the location of the transcriptional initiation site (9). The chicken rpL7a polypyrimidine tract is the shortest (8 bp) polypyrimidine tract so far determined for a ribosomal protein gene whereas the human rpL7a polypyrimidine tract at 25 bp (19) is the longest so far observed.

The use of more than one transcriptional start site is common to the three vertebrate rpL7a genes; two sites in mouse and human (19, 23) and three in chicken (Fig 1). In this property the rpL7a genes differ from most of the other rp genes which use only one transcriptional start site (10, 41, 42, 44-46). The mouse, human and chicken rpL7a genes also show a great similarity in the structure of their promoter region and in the length of their 3' untranslated region (UTR). It has been reported that sequences in and around the UTR of ribosomal protein genes are required for translational control (47-49) and can also be involved in translational regulation during development (39, 50). The chicken rpL7a gene contains a very short 3' untranslated leader (20-22 bp) and first exon (23-25 bp) which is a characteristic of the mouse and human rpL7a genes (19, 23) and other mammalian ribosomal protein genes. In all three genes the first intron begins directly after the first ATG.

A comparative sequence analysis of the 5' ends of the mouse, human and chicken rpL7a genes (Fig 2) has identified two elements (Box A and Box B) within the first 56 bp of 5' upstream sequence which are conserved over the 600 million years of divergent evolution that separates mammals and birds (51). The importance of these conserved sequence elements in promoter function was confirmed by in vitro and in vivo analyses. Only the conserved rpL7a sequence elements were found to bind protein factors from mouse L cell nuclear extracts (Figs 3 & 4). Transient transfection assays (Fig 6) showed that the minimal mouse rpL7a promoter region required for efficient expression of a reporter gene contains this region of maximum homology between the three promoters. Mutations located in Box A between nts -56 and -39 (Fig 2) abrogate promoter activity (Fig 6) and destroyed the ability to bind nuclear factors (Fig 3). Mutations within the Box A sequence define the upstream border of the minimal promoter required for efficient transcription of the mouse rpL7a gene as lying between nts -50 and -56. However the presence of a very low basal level of correctly initiated transcripts seen in cells transfected with deletions or mutations...
within Box A (data not shown) suggests that this element is involved in enhancing transcription as opposed to determining the position of the transcriptional start sites. To our knowledge the Box A sequences have not been identified as being important in the expression of other vertebrate genes. The second element, Box B, is located between nts -4 and -25 and covers a large conserved region between the three rpL7a promoter regions partially overlapping the polypyrimidine tracts of the human and mouse genes (Fig 2).

No relevance has been shown for the nt -95 to -60 region where a very strong homology has been found between the mouse and human rpL7a promoters but not with the chicken rpL7a promoter (Fig 2). Such a high degree of conservation suggests a possible importance of this region which is restricted to mammals. This sequence might be an element important during specific phases of the mammalian cell cycle or during differentiation or for increasing mammalian rpL7a gene expression after treatment with various inducing agents such as hormone (26) or UV (25). Another region downstream of the Box A element, between nt -35 and nt. -20, shows a lower degree of conservation between the three species (Fig 2). This is the region that contains a small A + T rich region in all three rpL7a genes. An A + T rich region has been detected in a number of other mammalian rp genes usually between 20-30 bp upstream from the start of transcription (10, 41, 42, 44-46). In the mouse S16 rp gene, the region containing this sequence has been shown to be important for the promoter efficiency and can be replaced by a canonical TATA box without affecting the rate of transcription (9). We did not detect the binding of any nuclear factors to the corresponding region of the mouse rpL7a gene in vitro, but it cannot be excluded that a weak interaction exists which can be stabilized in vivo by protein-protein interaction with the other factors involved in the expression of the rpL7a (i.e. Box A and Box B elements).

The mouse rpL7a gene shows a general architecture similar to a number of other mammalian rp genes which also contain the 5' border of their minimal promoter within a short distance (about 80 bp) upstream from their transcriptional start sites (5). However, the important sequence motifs present in the rpL7a Box A and Box B have not been detected in the five other mammalian rp promoters analyzed (4-11), nor have sequence elements found to be important for the expression of the five other mammalian rp genes been detected in the 5' upstream region of rpL7a. This suggests the absence of promoter elements common to all mammalian rp genes and that transcription of the various rp genes may be regulated by different transcription factors. Alternatively, the rp genes may be divided into different groups, each of which have a common set of promoter element transcription factor binding sites. The analysis of a much larger number of rp gene promoter elements is required to distinguish between these possibilities.

In conclusion only a small 5' upstream DNA sequence region is essential to drive the expression of the mouse rpL7a gene in vivo. This region can bind nuclear factors in vitro and its importance is indicated by its conservation over the 600 million years of evolution which separates mammals from birds. No similarity between the sequence of these rpL7a promoter elements and described promoters elements of other eukaryotic ribosomal protein genes has been found. Further studies will involve cloning of the protein factors that bind to the rpL7a promoter region as well as determining whether it is conserved in other lower eukaryotic rpL7a homologues.

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