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ISOLATION OF A CDNA CLONE ENCODING THE RNASE-SUPERFAMILY-RELATED GENE HIGHLY EXPRESSED IN CHICKEN BONE MARROW CELLS+

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The RNase gene superfamily combines functionally divergent proteins which share statistically significant sequence similarity. Known members assigned to this family include secretory and nonsecretory RNases; angiogenin; eosinophil cationic protein; eosinophil-derived neurotoxin; sialic-acid binding lectin and anti-tumor protein P-30. We report the cDNA cloning of the chicken **RN**ase **S**uper **F**amily **R**elated (**RSFR**) gene that is specifically overexpressed in normal bone marrow cells and bone marrow-derived AMV transformed monoblasts. It codes for a 139 amino acid protein with a putative signal peptide and remarkable conservation of active-site residues, other residues known to be important for substrate binding and catalytic activity and half-cystine residues common for all RNase family members. Phylogenetic tree analysis shows that RSFR defines a new group of genes within the family. We also conclude that an amino acid sequence block CKXXNTF(X)11C is a "shortest RNase superfamily signature" which is both necessary and sufficient to identify all previously recognized family members as well as chicken RSFR.

Similarities among amino acid sequences first noted by Gleich et al. (1) between human pancreatic RNase (HPR), eosinophil cationic protein (ECP or RNS3) and eosinophil-derived neurotoxin (EDN or RNS2) disclosed a group of proteins which comprise the ribonuclease gene superfamily. Another family member was postulated to be angiogenin (ANG) since its amino acid sequence is also similar to that of HPR, ECP (RNS3) and EDN (RNS2) (2-5). All of these genes

+Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. X64743 G gallus RSFR mRNA.

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code for polypeptides with remarkable conservation of three or four pairs of properly spaced cysteine residues, a number of other residues important for formation of the three-dimensional structure and with identical catalytic ribonucleolytic active site residues histidine-12, histidine-119 and lysine-41 (numbering according to HPR) (2-10).

An overall homology of EDN (RNS2), ECP (RNS3) and ANG to HPR is approximately 30%, and, although all three contain some ribonuclease activity (11-14), they appear to have additional distinguishable biological activities. Whereas ECP (RNS3) has been shown to stimulate factor XII-dependent coagulation (15), to inhibit the anticoagulant effects of heparin (16), to block lymphocyte proliferation induced by mitogen (17) and also to display a potent cytolytic and helminthotoxic activities (18), the only known biological activity of EDN (RNS2) is neurotoxicity: it induces ataxia and paralysis and can cause damage of myelinated neurons when injected intrathecally or intracerebrally into experimental animals (1), an effect known as the Gordon phenomenon. Angiogenin has no activities similar to those of eosinophil-derived members of the family, but it has unusual RNase activity specifically cleaving 28S and 18S rRNA (14). Although it is a potent stimulator of neovascularization in the chicken chorioallantonic membrane and rabbit cornea assays (19,20), the tissue distribution and developmental expression pattern of ANG mRNA is not temporally related to vascular development in the rat (21). These data indicate that while being structurally closely related, RNase superfamily genes may have divergent functions.

We report the cloning of RNase superfamily-related (RSFR) gene that is overexpressed in bone marrow cells of chickens. The evolutionary position of the RSFR gene on a phylogenetic Fitch tree shows that it defines a new group of RNase superfamily members closely related to but different from ECP(RNS3), EDN(RNS2) or canonical RNases.

MATERIALS AND METHODS

Cell lines, RNA preparations and Northern analysis. Total cellular RNA was isolated from chicken cell lines and tissues by the acid guanidinium thiocyanate method of Chomczynski and Sacchi (22). Northern hybridization with random-primer ^{32}P-labelled DNA probes was carried out as described in Sambrook et al. (23). Chicken cell lines used were BM2C3, a line of AMV-transformed myeloblasts (24), one stock of this line was kindly provided by M. Dvorak from J. Lipsick laboratory (Stony Brook, NY) and another stock from D. Stehelin (Pasteur Institute, Lille); BK3A and 293, ALV-induced bursal lymphoma cell lines (25) provided by M. Linial (Fred Hutchinson Cancer Research Center, Seattle); CHB1, a cell line of chicken fibroblasts transformed by myc-containing virus HB1 that was established in P. Neiman's laboratory; RPL1, Marek disease virus transformed T-lymphocytes (26) and 6C2 cell line of AEV-transformed erythroblasts (27) were obtained from T. Graf; HD11, MC29-transformed chicken macrophages (28) were obtained from M. Dvorak; chicken bone-marrow-derived cells (MOC20 and MOC13), thymus-derived cells T44 and spleen-derived cells b38 transformed by the MHE226 virus that encodes for the P135$^{gag\text{-}myb\text{-}ets}$ (29) were recently established by V. Laudet in D. Stehelin's laboratory. Primary chicken fibroblasts were prepared by standard procedure.

Isolation and sequencing of the RSFR clone. In the course of screening λgt11 expression cDNA library from BM2 cell line (kindly provided by A. Sippel) for DNA-binding nuclear factor CTCF (30) by probing with the ^{32}P-labelled recognition site DNA probe, we did not discard fortuitously picked clones (i.e. those which did not code for any sequence-specific DNA-binding activity), but saved them in order to test whether any of them might have interesting tissue-specific expression patterns.

By screening 12 cDNA clones which did not cross-hybridize to each other, we found one clone (clone #4) that hybridized strongly to BM2 RNA species. Clone #4 was subcloned into the pBluescript vector and both of its DNA strands were sequenced by the dideoxy chain termination

technique as described by manufacturers of the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Ohio).

Protein Data Bank Analysis. The amino acid sequence of RSFR was compared with SWISSPROT version 20 (31) and version 4.0 of the BLOCKS database (32) using PATMAT search software (33). The pancreatic ribonuclease family signature motif used to search SWISSPROT was obtained from release 8.0° of the Prosite dictionary (34). Following alignment of related sequences using the Clustal V software package, evolutionary distances between all possible pairs were calculated and corrected distance matrix was used to determine the phylogenetic tree by the Fitch Least Square Analysis according to Laudet et al (35).

RESULTS

Twelve cDNA clones from the BM2 cell λgt11 cDNA library were selected to probe Northern blots of different chicken cells. Clone #4 was found to be the only one that hybridized to the RNA from BM2 cells, detecting a highly expressed RNA message of about 1.1 kbp (Fig. 1, lane 8). It was also positive with the total RNA from embryonic spleen (lane 1) but negative with RNA from transformed erythroid cells (lane 6), macrophages (lane 9) and T cells (lane 10), indicating that clone #4 could code for a gene expressed in a tissue-specific fashion. In addition, clone #4 DNA probe did not detect 1.1 kbp mRNA in any of the MHE226-transformed cells tested (lanes 2-6). Exept in erythroblasts (lane 7), relatively weak ubiquitous RNA bands were also detected indicating either expression of highly related sequences or unspliced intermediates of the 1.1kbp RNA. We decided that non-random pattern of the clone#4 expression is a sufficient reason to characterize this clone in detail and have sequenced it.

Figure 2 shows that cDNA clone #4 contains one 417 bp long open reading frame beginning at ATG within the NcoI site with perfect Kozak translation initiation sequence . The deduced 139 amino acid sequence was compared to ca. 23,000 SWISS-PROT protein sequences using PATMAT

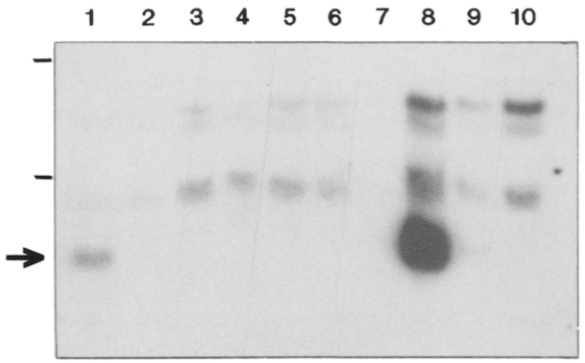

FIG. 1. Northern blot analysis of mRNA for clone #4. Total RNA samples were prepared from chicken 10 day old embryonic spleen (lane 1); bone marrow derived MHE226 -transformed MOC20 and MOC13 cells (lanes 2 and 3); MHE226-transformed cells T44 derived from thymus (lane 4); MHE226-transformed cells R38 derived from spleen and grown in low-serum (lane 5) or high-serum (lane 6) conditions; AEV-transformed erythroblasts 6C2 (lane 7); AMV-transformed myeloblasts BM2C3 (lane 8); MC29-transformed macrophage-like cell line HD11 (lane 9); RPL1 cell line of the Marek disease virus transformed chicken T-lymphocytes (lane 10). All lanes contained reasonably equal amounts of loaded RNA as judged by ethydium bromide staining of the gel and did not show any RNA degradation products when the same blot was hybridized to three other different probes (data not shown). Positions of 18S rRNA (2.0kbp) and 28S rRNA (4.2kbp) are marked at the left side of the blot; arrow head indicates 1.1kbp RSFR mRNA.

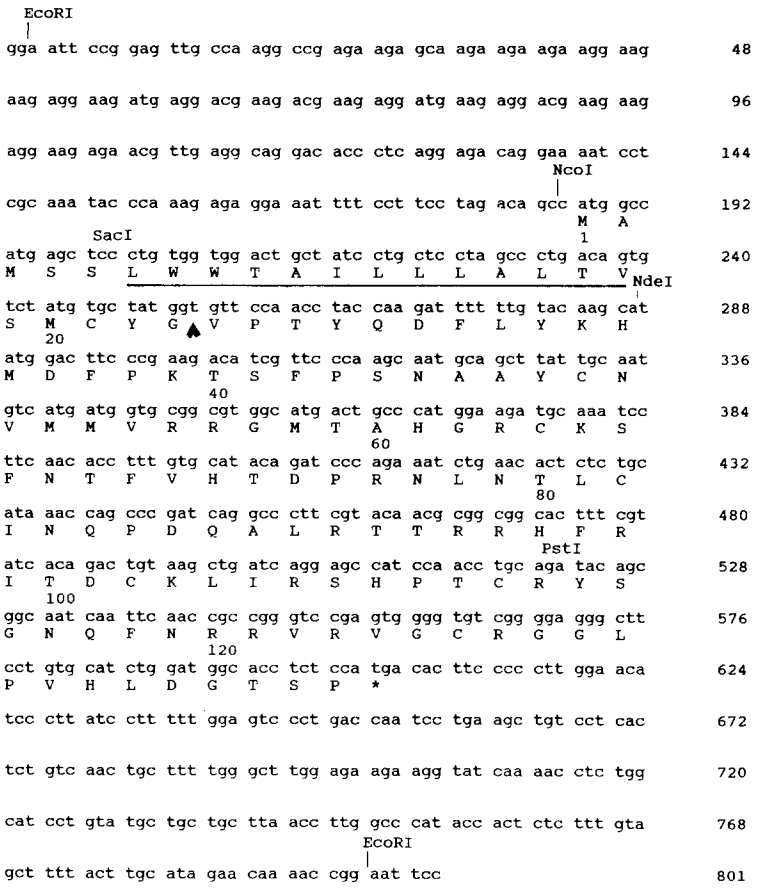

FIG 2. Complete nucleotid sequence of the cDNA clone #4 and deduced amino acid sequence of the RSFR. Both EcoR1 sites are from the pBluescript vector. Hydrophobic residues of the putative leader sequence are underlined. Arrow head shows potential cleavage site at consensus found by von Heijne's rules (36).

search software. The highest scoring sequences were angiogenin and ribonuclease, matching sixteen amino acids in a search window of thirty for *C. serpentina* (snapping turtle) ribonuclease and thirteen amino acids out of thirty for mouse angiogenin precursor.

Fig. 3 shows the following major distinctive homologies between RSFR and other members of the family:

(1) Conservation of identical residues His-12, Lys-41 and His-119 (numbering according to RNase A (37) which were identified by numerous workers (see refs. in (37)) as catalytically active residues;

(2) Apparent alignment of the half-cystine residues important for folding and conservation of the three-dimensional structure;

(3) Alignment of 8 out of 10 residues involved in substrate binding.

Although there are noticeable and potentially important differences between amino acid sequences of the RSFR and other family members, the sequence alignment demonstrates that the chicken RSFR belongs to the RNase superfamily. Fig.3 has also clearly indicated that SABP, the sialic-acid binding lectin from frog eggs (38) and anti-tumor protein P-30 from *Rana pipiens*

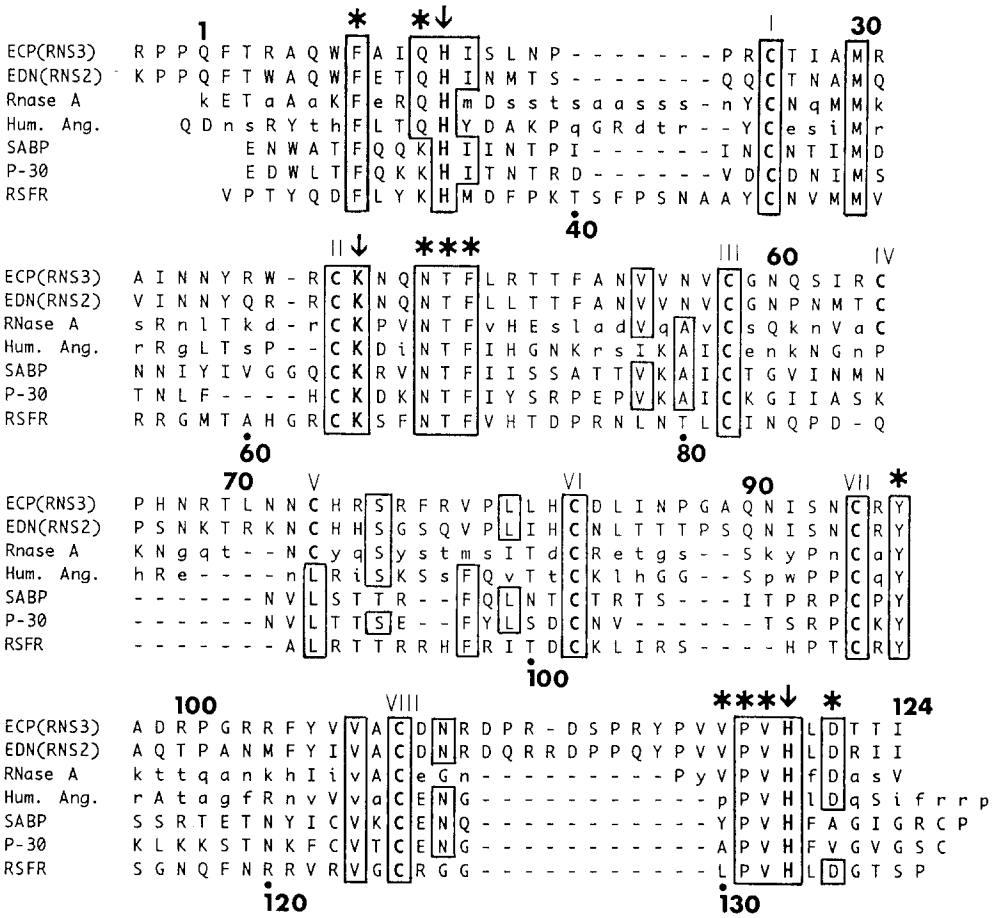

FIG 3. Comparison of the amino acid sequences of ECP(RNS3), EDN(RNS2), RNase A, human ANG, sialic acid binding protein (SABP), anti-tumor protein P-30 and RSFR. Upper line positions are numbered according to the residue number of RNase A (37), whereas in lower line according to RSFR. Capital letters in the RNase A sequence indicate residues that are conserved in at least 38 of 39 mammalian pancreatic RNases (38); capital letters in the human ANG show residues that are also conserved in bovine (3) and mouse (5) angiogenins. Identical residues in more than 4 out of 7 sequences are enclosed in boxes; catalytic histidine and lysine residues shown by arrows; conserved cysteine residues shown in bold letters. Substrate-binding residues are marked by asterisks. Roman numerals are used to designate half-cystines forming disulfide bonds between pairs I-VI, II-VII, III-VIII and IV-V (37). Dashes represent gaps introduced to permit alignment of the catalytic residues and cysteines.

early embryos (39) also belong to the RNase gene superfamily. The later protein was shown to have a substantial antitumor effect in vivo (40) and is carrently undergoing clinical trials in the United States (39). Fig.4 shows the phylogenetic tree of the superfamily members.

Fig. 5 shows that RSFR is predominantly detected in bone marrow (lane 11) where its expression level is practically equal to that in AMV-transformed myeloblasts BM2C3 (lanes 9,10). Relatively much lower level of RSFR mRNA was detected in spleen (lane 6) and at even lower levels in thymus (lane 1), bursa of Fabricius (lane 2) and liver cells (lane 3). In addition to negative cell lines shown in Fig. 1, RSFR was not detectable in muscle (lane 4); brain (lane 5); kidney (lane 7); heart (lane 8); and also in myc-transformed fibroblast line CHB1 (lane 12); and ALV-induced bursal lymphoma lines BK3A and 293 (lanes 13,14). Also there was no RSFR expression

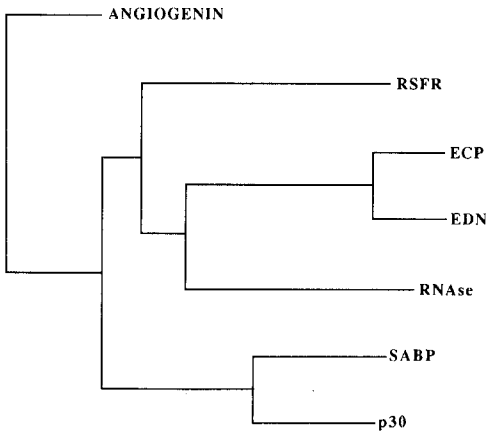

FIG.4. Unrooted phylogenetic Fitch Least Square Tree clustering RNase superfamily genes. Construction of the tree was based on the CLUSTAL V alignment. Horizontal distance between individual genes coresponds to their evolutional divergence. NBRF accession numbers of sequences shown are: ANGIOGENIN, NRHUAG; ECP, B35328; EDN, A35328; RNase, A32471; SABP, A27121; p30, A39035.

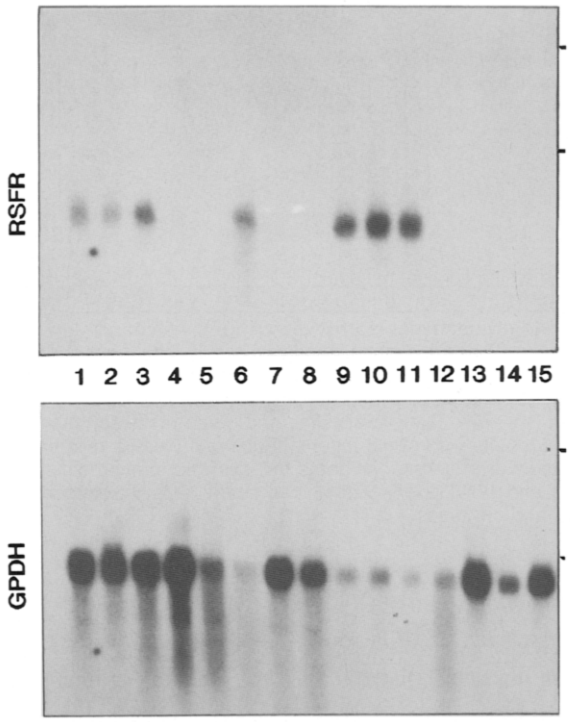

FIG .5. Differential expression of the RSFR gene. Total RNA samples were from chicken thymus (lane 1); bursae (lane 2); liver (lane 3); muscle (lane 4); brain (lane 5); spleen (lane 6); kidney (lane 7) and heart (8); from BM2C3 myeloid cell line obtained from Lipsick's laboratory (lane 9) or from Stehelin's laboratory (lane 10); bone marrow (lane 11); HB1-transformed fibroblast cell line CHB1 (lane 12); ALV-induced bursal lymphoma cell lines BK3A (lane 13) and 293 (lane 14); primary culture of chicken embryonic fibroblasts (lane 15). The same blot was hybridized first to the clone #4 probe (top panel) and then to the chicken glyceraldehyde 3-phosphate dehydrogenase gene (GPDH) (bottom panel). Note that in lanes 6 and 9-12 there was at least 10 times less RNA loaded than on other lanes. Positions of rRNAs are shown at the right side of the blots.

detected in primary embryonic fibroblast culture (lane 15). Taken together, data of Figs. 1 and 5 suggest that RSFR is preferentially expressed in precursor cells of the myeloid lineage.

DISCUSSION

We have isolated a cDNA clone for a chicken gene closely related to the RNase superfamily therefore called RSFR. It codes for 139 amino acid protein that possess all major features characteristic for primary structure of other previously identified members of this family. A perfect alignment was obtained (Fig. 3) not only for half-cystine residues (which form disulfide bridges in the folded molecule) and for active-site residues His-12, His-119 and Lys-41, but also for other residues potentially important for substrate binding.

In order to elucidate relationship of the RSFR to other members of the RNase gene superfamily we have constructed the phylogenetic Fitch tree for these genes. Fig.4 shows that RSFR gene defines a new group of the RNase family members, most closely related to but different from ECP, EDN and RNase A . This may well indicate that there is a mammalian homologous of the chicken RSFR gene.

We have also noticed that the pancreatic ribonuclease family signature CKXXNTF suggested by Bairoch (34) is not sufficient to identify all and only meembers of the RNase superfamily, since this motif allows the inclusion of red clover necrotic mosaic virus, revealed by a motif search of the SWISS-PROT database. Extending this motif upto CKXXNTF(X)11C creates an absolutely specific consensus sequence common for all and only members of this family. Therefore, we propose to call the CKXXNTF(X)11C motif "the shortest RNase superfamily signature".

Although we do not know whether RSFR protein is co- or post-translationally processed, it is worth noting that its N-terminal segment is markedly hydrophobic (including an uninterupted stretch of 13 sequential hydrophobic residues interspersed by 7 aliphatic residues as marked in Fig. 2) a characteristic of a leader sequence common for RNases, angiogenin and EDN (RNS2) (6). The putative signal-sequence cleavage site shown in Fig. 2 was determined by the von Heijne's rules (36). If it is a functioning site actually used by the signal peptidase, then length of the RSFR leader sequence would be 23 amino acids, comparable to 25 for RNase A (41) and angiogenin (5) or 27 for ECP(RNS3) (8) and EDN(RNS2) (6).

Although RSFR and other RNase superfamily members share important primary structural features, they are very unlikely to have common functions. A comparison of their expression patterns supports this conclusion. Indeed, among normal chicken tissues tested, a predominant site of the RSFR expression appears to be the bone marrow (Figs. 1 and 5). Consistent with these data, only bone-marrow-derived myeloid cell line, BM2C3, was found to be expressing RSFR mRNA at high levels (Figs. 1 and 5). Since level of the RSFR expression is equally high in both normal bone marrow cells and in v-myb induced BM2C3 cell line (Fig.5), RSFR gene is unlikely to belong to the group of genes specifically responsive to myb. The presence of the putative signal peptide in the RSFR protein and specific pattern of expression suggest that RSFR gene could encode a component of promyelocyte granules. Neither EDN(RNS2) or ECP(RNS3) were detected in the sample of total RNA from bone marrow cells (9,10); angiogenin is easily detectable in total RNA from liver, but no angiogenen mRNA is detectable in total RNA from nonhepatic tissues of neonatal or adult rat (21). There is no data on tissue distribution of SABP, sialic acid binding lectin

purified from frog (*Rana catesbeiana*) eggs (38). We also did not come across the data on expression patterns for anti-tumor P-30 protein (39).

What could be a function of the RSFR? To address this question, we have to define the exact nature of RSFR producing precursor cells in bone marrow and we also plan to take an advantage of working with chicken model which provides an opportunity to express RSFR in many chicken cells by replication-competent retroviral constructs. After completing this manuscript, we also checked for potential DNA sequence homologies between RSFR gene and GenBank release 70 DNA sequences and noticed that only 15% of the RSFR amino acid sequence is different from unpublished sequence for chicken RNase A/angiogenin protein submitted by T.H. Graf and T.Nakano under the entry number X61192. The most noticebale difference includes a six consequtive amino acid substitution of the most positively charged domain of RSFR at positions 94-98 (Fig.3) for uncharged amino acids. Significance of this difference could be tested by direct functional comparison of RSFR and chicken RNase/angiogenin genes.

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