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Abstract

In the chicken, a high degree of heterogeneity at the protein level has been reported for the creatine kinase-type B (B-CK). Here we show that the two B-CK isoproteins, Ba- and Bb-CK, are encoded by two mRNAs, which are derived from a single copy gene by a stochastic alternative splicing mechanism. The transcription of the single hnRNA is directed by a complex promoter region containing a stretch of sequences which is highly conserved among all the B-CK genes known to date. This stretch encompasses a putative binding site for the TA-rich DNA-binding protein (Hobson, G. M., Mitchell, M. T., Molloy, G. R., and Pearson, M. L. (1988) Nucleic Acids Res. 16, 8925-8944) which is located in the distal part of the promoter region, while the proximal portion containing the TATA-box used in vivo is not conserved between chicken and mammals. The two isoproteins arising from this gene contain distinct N-terminal portions. According to comparative analysis, Bb-CK is the form which is homologous to the mammalian B-CKs, whereas Ba-CK shows some sequence features unique among all other vertebrate cytosolic creatine kinases characterized […]

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A Unique Chicken B-creatine Kinase Gene Gives Rise to Two B-creatine Kinase Isozymes with Distinct N Termini by Alternative Splicing

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In the chicken, a high degree of heterogeneity at the protein level has been reported for the creatine kinase-type B (B-CK). Here we show that the two B-CK isozymes, Ba- and Bb-CK, are encoded by two mRNAs, which are derived from a single copy gene by a stochastic alternative splicing mechanism. The transcription of the single hRNA is directed by a complex promoter region containing a stretch of sequences which is highly conserved among all the B-CK genes known to date. This stretch encompasses a putative binding site for the TA-rich DNA-binding protein (Hobson, G. M., Mitchell, M. T., Molloy, G. R., and Pearson, M. L. (1988) Nucleic Acids Res. 16, 8925-8944) which is located in the distal part of the promoter region, while the proximal portion containing the TATA-box used in vivo is not conserved between chicken and mammals. The two isozymes arising from this gene contain distinct N-terminal portions. According to comparative analysis, Bb-CK is the form which is homologous to the mammalian B-CKs, whereas Ba-CK shows some sequence features unique among all other vertebrate cytosolic creatine kinases characterized so far.

The creatine kinases (EC 2.7.3.2) catalyze the reversible exchange of high energy phosphate between ATP and creatine. In vertebrates, three distinct groups of subunits, each showing its characteristic developmental regulation and tissue-specific distribution, have been described: the skeletal muscle specific M-CK, which is also expressed in mammalian heart, B-CK, which is expressed in neuronal and smooth muscle tissues, in heart, B-CK, as well as in many embryonic cell types, and the mitochondrial form Mi-CK, the expression of which seems to be restricted to the tissues that also express a cytosolic form. While the cytosolic forms, M- and B-CK, are active as dimers, the mitochondrial subunits are bound as octamers to the outer surface of the inner mitochondrial membrane (Schlegel et al., 1988a; Snyder et al., 1988). It has been proposed that these enzymes constitute a phosphorylcreatine shuttle (Bessman and Geiger, 1981; Tomes and Shapiro, 1985; Wallimann and Eppenberger, 1985; Wallimann et al., 1989), i.e. a high energy phosphate back-up and transport system, which is especially important in tissues which have to meet sudden changes in energy demand. Several cellular structures that carry out energy consuming processes have been shown to specifically bind or be functionally coupled to active forms of CK and thus have been defined as terminals of the phosphorylcreatine shuttle system. Examples include myofibrils (Schäfer and Perriard, 1988; Wallimann et al., 1983), the Ca++ ATPases of the sarcoplasmic reticulum (Rossi et al., 1990), brain synaptic plasma membranes (Lim et al., 1983), acetylcholine receptor-rich membranes (Wallimann et al., 1985), as well as the mitotic spindle (Cande, 1983; Koons et al., 1982) and the polysomes (Savabi et al., 1988) of certain cell types.

The regulation of CK expression was studied extensively with a special emphasis on the isozyme switch from B-CK to M-CK during terminal differentiation of skeletal muscle tissues (Eppenberger et al., 1964; Perriard et al., 1986) and in myogenic cell cultures (Caravatti et al., 1979; Morris et al., 1972; Turner et al., 1974). This isoenzyme transition has been shown to be regulated at the transcriptional level (Kwiatkowski et al., 1985; Perriard, 1979; Rosenberg et al., 1982). Furthermore, the expression of both cytosolic isoforms is modulated by hormones. While M-CK activity is responsive to somatomedins (Ewton and Florini, 1981), B-CK appears to be regulated by estrogen (Reiss and Kaye, 1981), by parathyroid hormone and prostaglandin E2 (Sömjen et al., 1984a), as well as by 24R,25-dihydroxyvitamin D (Sömjen et al., 1984b).

Further heterogeneity at the protein level could be demonstrated for the cytosolic forms (Rosenberg et al., 1981) as well as the mitochondrial type CK of chicken (Hossle et al., 1988; Schlegel et al., 1988b) and human. The multiple M-CK subtypes must be due to posttranslational modifications, since unique transcripts derived from single copy genes could be found in all species examined (Bentfeld et al., 1988; Jaynes et al., 1986; Trask et al., 1988). In contrast, there is evidence for at least two Mi-CK transcripts derived from distinct genes in chicken (Hossle et al., 1988) as well as in human (Haas et al., 1989) and for two mRNAs coding for B-CK subtypes with distinct isoelectric points, the acidic Ba-CK and the basic Bb-CK, in chicken (Perriard et al., 1987).

In chicken, two B-CK cDNA clones, 114 (Hossle et al., 1986) and 18c, have been isolated. While H4 contains the whole protein coding region for Bb-CK (Hossle et al., 1986; Perriard et al., 1986), the transcription of the single hRNA is directed by a complex promoter region containing a stretch of sequences which is highly conserved among all the B-CK genes known to date. This stretch encompasses a putative binding site for the TA-rich DNA-binding protein (Hobson, G. M., Mitchell, M. T., Molloy, G. R., and Pearson, M. L. (1988) Nucleic Acids Res. 16, 8925-8944) which is located in the distal part of the promoter region, while the proximal portion containing the TATA-box used in vivo is not conserved between chicken and mammals. The two isozymes arising from this gene contain distinct N-terminal portions. According to comparative analysis, Bb-CK is the form which is homologous to the mammalian B-CKs, whereas Ba-CK shows some sequence features unique among all other vertebrate cytosolic creatine kinases characterized so far.
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et al., 1987) 18c lacks the C-terminal part of the coding region and differs from H4 in the 5'-untranslated region and in the N-terminal portion of the coding region (see Fig. 1A). In order to understand how the mRNAs coding for Ba- and Bb-CK are generated and to get sequence information about the Ba-CK protein, we decided to clone the chicken B-CK gene. Furthermore, the resulting sequence information should enable us to investigate B-CK transcription and its regulation by hormones and during myogenesis, as well as the different tasks of the two isoproteins with respect to the energy metabolism of the B-CK expressing cells.

Here we report that a single copy B-CK gene gives rise to two variants of B-CK corresponding to the cDNA clones H4 and 18c by a stochastic alternative splicing mechanism and that Ba-CK is encoded by the 18c-type transcript. The two resulting proteins have distinct N-termini, and that of Ba-CK shows some sequence features which are unique among all cytosolic vertebrate Cks known to date.

MATERIALS AND METHODS

Isolation and Cloning of Genomic B-CK Sequences—A chicken genomic DNA library was kindly provided by Dr. M. Ballivet of the University of Geneva (Switzerland). This library contains partially Mbol-digested DNA from erythrocytes of White Leghorn chicken cloned into the BamHI site of the bacteriophage λ cloning vector λ47 (Loenen and Brammar, 1980) which was propagated using E. coli strain Q359 as a host. Approximately 4.7 × 10^11 plagues were screened at a density of 5 × 10^9 plaques/plaque (100 × 100 mm).

The plaque lifts were performed with biopsy nylon membranes and hybridized with the 32P-labeled 1400 base pair (bp) EcoRI fragment from the chicken B-CK cDNA clone H4 (Hosseil et al., 1986) at 65°C using the conditions suggested by the manufacturer (Pall, EAST HILLS, NY). The filters were washed four times for 30 min at 65°C again as indicated by the supplier of the membrane.

The polymerase chain reactions for the isolation of the middle part of the gene were carried out as described earlier (Sakai et al., 1988). Genomic DNA from blood of two White Leghorn chicken inbred strains (number 2854 of family 7708 and number 2883 of family 8708) kindly provided by Dr. C. Haggar from the Swiss Federal Institute of Technology in Zurich was prepared as previously described (Jeanpierre, 1987). One mg of DNA was used as template/assay and 35 cycles were carried out using the following conditions: denaturation: 1 min, 95°C; annealing: 2 min, 60°C; elongation: 6 min, 70°C. All oligodeoxynucleotides were synthesized on a Gene Assembler (Pharmacia LKB Biotechnology Inc.) and the Taq-polymerase was from New England Biolabs.

Restriction Mapping and Southern Blots—The restriction maps of the genomic DNA were analyzed by agarose-gel electrophoresis and transferred to Biodyne nylon membranes (Pall). Thereafter, UV cross-linking and hybridization with 32P-labeled probes was performed according to the method of Church and Gilbert (1984).

Production of Radiolabeled Probes—The desired, vector-free fragments were isolated after digestion and agarose gel electrophoresis with the Gene Clean kit (Promega). Twenty-five to 40 mg of DNA were labeled with [α-32P]dCTP (6000 Ci/mmol) from American Pharmacia by the random-primed labeling method described previously (Feinberg and Vogelstein, 1984), with yields in the range of 3 × 10^6 to 5 × 10^7 cpm/pm.

Cloning Procedures—All genomic fragments were subcloned in pBR322 (Stratagene) and plasmid vectors in Dihibonics K5 plasmid vectors (Stratagene). The chicken cDNA clone TW18-1 was constructed by deletion of the 3'-terminal 240-bp BamHI fragment from clone J3-5, which contains the cDNA 18c cloned into the EcoRI site of the vector pSP65. This fragment was replaced by a 1050-bp BamHI fragment from clone B10-1, which corresponds to the 3'-part of the chicken B-CK (Hosseil et al., 1986). All plasmids were grown in E. coli X-L1 Blue (Stratagene), and DNA was isolated by an alkaline lysis-type method (Maniatis et al., 1982).

Sequence Determination—Plasmid sequencing has been performed according to the dideoxynucleotide chain-termination method (Sanger et al., 1977), using synthetic primers directed against B-CK sequences or overlapping subclones, which were hybridized with the universal primers directed against vector sequences flanking the inserted fragments. The reactions were carried out as suggested by the suppliers of the systems used, Sequenase (USB) and the Taq-Track Deaza kit (Promega).

S1-nuclease and Primer-extension Analysis—Primer-extension analysis were carried out using the oligodeoxynucleotides 040788-79 for the 3'-proximal portion of the 5'-terminal part of the transcribed H4-type transcript. The probes were 32P-labeled with T4 polynucleotide kinase as indicated by the manufacturer (Amersham Corp.) and a specific radioactivity of 4.5 × 10^6 cpm/pmol were obtained. 0.4 pmol of the probes and 2 μg of poly(A)+ RNA were copurified with ethanol, dried, and the pellets were resuspended in 10 μl of 375 mM KCl, 10 mM Tris-HCl, pH 7.5, boiled for 2 min and incubated at 50°C (040789-79) or at 60°C (280688-75) for 2 h. Then, the reactions were adjusted to 0.5 mM each dATP, dCTP, dGTP, and dTTP, 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, 3 mM MgCl2, 10 μg/ml actinomycin D; finally 20 units of RNAsin (Promega) and 60 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) were added, and the reactions were carried out at 37°C for 1 h in a total volume of 50 μl. The reactions were stopped by resuspending to 0.2 μl NaOH, 10 mM EDTA in 100 μl and incubating it for 1 h at 42°C. Finally the mixtures were neutralized with 43 μl of 0.3 M Tris-HCl, pH 7.5, 0.5 mM acetic acid, 1 μM NaCl, extracted once with volume phenol/chloroform (1:1), precipitated with ethanol, and electrophoresed on a 7% urea, 10% acrylamide sequencing gel. S1 analysis were carried out by a modification of a method described earlier (Weaver and Weissman, 1979). Both probes utilized were synthetic oligonucleotides: 120589-46 (49 to +58 with respect to the genomic sequence, and 11 nucleotides of extraneous sequence were added at the 3' end) for the localization of the transcription start site, and 090589-40 (41 to +76 and +71 to 787 with respect to the genomic sequence plus 11 extraneous nucleotides at the 3' end) for the characterization of the 5' end of the H4-type transcript. End-labeled probe (10 fm, 4.5 × 10^6 cpm/pmol) was copurified with RNA (20 μg), dissolved in 18 μl of 80% (v/v) formamide, 10 mM PIPES, pH 6.4, 0.02% sodium dodecyl sulfate, 1 mM EDTA, 0.5 mM NaCl, heated to 80°C for 5 min, and incubated at 55°C (120589-46) or at 48°C (090589-40) overnight, respectively. Subsequently, the mixes were chilled on ice, 250 μl of 1M NaCl, 30 mM sodium acetate, pH 4.5, 5 mM ZnSO4, 30 units of S1 nuclease from Boehringer Mannheim) were added and incubated for 1 h at 37°C (120589-46) or 46°C (090589-40). Protected fragments were analyzed on 7% urea 10% acrylamide sequencing gels. Gels were exposed to x-ray films at −70°C, together with enhancing screens for 2-12 h.

Production and Analysis of In Vitro Generated B-CK Proteins—Linearized pSP65 clones containing the H4 Bb-CK or TW18-1 Ba-CK insert were transcribed in vitro as previously described (Melton et al., 1984) using SP6 RNA polymerase (Boehringer Mannheim) and 40 units of RNase inhibitor RNAsin (Promega). The further treatment of the transcripts as well as the translation in rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (Amersham Corp.), the immunoprecipitations, the sample preparation and the two-dimensional gel electrophoresis were carried out exactly as already described (Soldati et al., 1990).

RESULTS

Isolation of Genomic B-CK Sequences—In chicken, two B-CK-type cDNA clones, H4 (Hosseil et al., 1986) and 18c (Perriard et al., 1987), diverging in their 5' terminal portions, have been characterized (Fig. 1A). H4 contains the whole protein coding sequence as well as 32 nucleotides of the 5'-untranslated leader while 18c contains 60 nucleotides of leader and only the 5'-half of the protein coding region. The middle segment of H4 is identical to the 3' proximal portion of the coding region of 18c; in addition, the 5' terminal 12 bp of the cDNA H4 are also identical to a sequence located in the 5' noncoding sequence of 18c. S1 nuclease protection analysis revealed that the 10 nucleotides at the very N terminus of H4 described earlier (Hosseil et al., 1986) arose by an artifact that very likely occurred during cDNA synthesis (not shown). The presentation of the cDNA H4 and the numbering of the nucleotide positions in the sequence H4 as
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Fig. 1. A, comparison of the two B-CK cDNA clones H4 and 18c. Hatched boxes indicate sequences in the “nonoverlapping” regions of the two cDNAs, the dotted boxes indicate sequence identity between the two clones, the black box represents an 18c-specific sequence, and the empty box stands for H4-specific sequences. H4 contains a complete protein coding region from the start codon (AUG) at nucleotide 33 to the stop codon (UAA) at nucleotide 1176. 18c carries the coding information for only the N-terminal half of the respective protein and the putative start codon is at nucleotide 60. The BamHI restriction site (B) in the common regions of the two cDNAs has been used for the construction of the chimaeric cDNA clone TW18-1 (see text). B, the restriction map of the genomic B-CK locus of chicken was derived from the analysis of fragments isolated from X clones (T8, T56, and T62) and of a fragment obtained by amplification experiments (PCR, see below). The thick lines (upper part) indicate restriction fragments that hybridize to the two cDNA clones shown in A. The hybridizing fragments are shown in more detail in the lower part of the graph. The exons are indicated as boxes, which display patterns that are indicated in the map are: H, HindIII; R, EcoRI; B, BamHI; K, KpnI; X, XhoI, and T, TaqI (only for the expanded part of the map). Below the expanded part of the map, the four fragments obtained by the amplification experiments (PCR) are shown as black bars. The numbers beside them indicate the designations of the BGPCR primers utilized for the different amplifications, primer 1 being the 5’ most and primer 4 being the 3’ most. Furthermore, the sequencing strategy is shown (arrows). Multiple parallel arrows indicate sequencing of the respective region on several subclones.

In order to isolate genomic B-CK fragments a 3 L47 library of chicken erythrocyte DNA was screened using the cDNA clone H4 as a probe. With this approach we hoped to identify clones carrying H4-specific as well as 18c-specific genomic sequences. 4.75 x 10^6 plaques were analyzed by plaque hybridization and four independent positive clones, T5, T56, T62 (Fig. 1B), and T6 were isolated. Restriction mapping and Southern blot analysis revealed that T6 and T8 contain the same B-CK-specific coding sequences corresponding to the 3’ part of the gene, whereas T56 and T62 contain the 5’ end of the gene. While T56 extends in the 5’ direction 10 kb beyond the 5’ most HindIII fragment hybridizing to the cDNA clone H4, T8 contains 9 kb of genomic sequences downstream of the 3’ end of the gene, but unfortunately the two pairs of clones do not overlap with each other. Further analysis of T6 revealed that in this clone a rearrangement during cloning must have taken place. The vector portion of T6 was heavily altered, and its propagation was affected, which made it very difficult to get usable quantities of DNA to analyze this clone. We therefore decided not to further characterize T6.

In addition, the B-CK positive clones were clearly under-represented in the library we have utilized. A single copy gene such as the acetylcholine receptor a-subunit gene was shown to give rise to about five independent clones/million plaques screened from the same library. The B-CK clones had a frequency of one clone/1.2 million plaques, which is about six times less.

We assume that the sequences of the missing middle part of the gene are responsible for the rearrangements in the clone T6, as well as for the underrepresentation of the B-CK clones in the library. Additional screenings with fragments from either side of the gap were also unsuccessful. Therefore, we decided to utilize an alternative method for the isolation of the missing sequences, allowing us to circumvent the initial cloning steps necessary for the construction of a λ library.

According to genomic Southern blot analysis (see Fig. 6), the missing part of the gene (B-CK gap = BG) was predicted to be about 3 kb in length. This prompted us to design a strategy for the amplification of this sequence by the polymerase chain reaction (PCR, see Fig. 1B). Four oligonucleotides were synthesized (BGPCR1, 2, 3, and 4). BGPCR1 and 4 are derived from intron sequences flanking either side of the gap, while the primers BGPCR2 and 3 are derived from exon sequences, which are located on the missing part of the gene. Sequence analysis of the exons flanking the gap revealed that 290 bp corresponding to the middle part of the cDNA clone H4 (514-809), must be encoded by the missing fragment. We speculated that these cDNA sequences would be present in two exons of about 125 and 170 bp length. These assumptions were justified by the fact that all the locations of the splice junctions within the chicken gene with respect to the protein coding portion of the cDNA sequence H4 are conserved compared with those characterized within the homologous B-CK genes in rat (Benfield et al., 1988) and human (Mariman et al., 1987). While the primer BGPCR2 represents a plus strand sequence of the postulated exon in the 5’ part of the gap, primer BGPCR3 is designed to be a minus strand sequence of the other postulated exon.

With these four BGPCR primers, 35 cycles of amplification were carried out, using as the starting material 1 μg of high molecular weight genomic DNA from two inbred strain chickens of different White Leghorn families. We were able to amplify four distinct fragments with the primer combinations 1-4, 1-3, 2-3, and 2-4 (Fig. 1B) from both DNAs. The amplification 1-4 gave rise to a 3.1-kb fragment, which is in the expected range for the whole middle part including the sequences that overlap with the λ clones on either side. The length of the gap itself is 2.9 kb. The amplifications 1-3, 2-3, and 2-4 yielded fragments of 2.3, 1.6, and 2.4 kb, respectively. No differences could be found between the amplification products derived from the DNAs of the two inbred strains. In order to identify these amplification products as B-CK sequences, we performed a Southern blot analysis, using the cDNA clone H4 as a probe (not shown). The four fragments gave rise to unambiguous signals, which led us to conclude that all these sequences were part of the B-CK gene. Furthermore, we isolated the amplification product 1-4 and used this as a template for polymerase chain reaction assays with the primer combinations 1-3, 2-3, and 2-4. These amplifications gave rise to the same fragments as found when genomic DNA was used as template. Therefore, it is likely that all the fragments produced by these amplification experiments are

\[ \text{M. Ballivet, personal communication.} \]
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Tors, which allowed us to sequence the two exons with their flanking regions as well as the sequences overlapping with the \( \lambda \) clones. All sequences have been read up to five times on independent clones in order to make sure that mutations introduced by the inaccuracy of Taq polymerase could be unambiguously identified. The two exon sequences matched perfectly to the respective sequences of the cDNA clone \( \text{H4} \) and they are both flanked by canonical splice acceptor and donor sequences (Mount, 1982). In addition, the 5'-terminal as well as the 3'-terminal sequences of the fragment 1–4, which spans the whole middle part, are identical to the sequences deduced from the flanking \( \lambda \) clones.

Two Transcripts Corresponding to the cDNA Clones \( \text{H4} \) and 18c Arise by an Alternative Splicing Mechanism—Sequence analysis of the genomic fragments that hybridize to the cDNA probes (Fig. 1B) revealed that exons coding for the cDNAs \( \text{H4} \) and 18c are intermingled (Fig. 1B, 8). In light of the fact that \( \text{H4} \) codes for a B-CK protein (Hossle et al., 1986) and that \( \text{H4} \) as well as 18c have been shown by S1 analysis (Perriard et al., 1987) to correspond to mRNAs expressed in B-CK-containing tissues, we concluded that these two cDNA clones correspond to two B-CK mRNAs arising from one gene by an alternative splicing mechanism. The two alternatively spliced exons 2a and 2b span the parts of the two cDNAs between the regions of sequence identity (Fig. 1A, black and white boxes). The H4-specific exon 2b (Fig. 3, nucleotides 771–983) contains the cDNA sequence from nucleotide 13 to 225 and the 18c-specific exon 2a (Fig. 3, nucleotides 1380–1567) from 51 to 238 of the respective cDNA. Since the two exons contain the two putative translation start sites (Fig. 1A), the two mRNAs code for two B-CK protein species with distinct N termini (see below).

In addition to this feature, which is unique among all CK genes characterized so far, the chicken B-CK gene is about 9 kb long, which is almost three times the length of the mammalian B-CK genes. The lengths and the number of the exons/transcript, as well as the location of the splice sites in the gene with respect to the protein coding regions of the cDNA sequences, however, are almost identical to those in the mammalian genes. Only the exons 1, 2a, 2b, and 8 differ in length by some base pairs due to species-specific differences in the 5'-untranslated leader and the 3' trailer sequences, respectively. All exons are flanked by canonical splice sites, and potential lariat acceptor sequences could be found in the expected locations near the splice acceptor sites.

Characterization of Exon 1 and Localization of the Transcription Initiation Site—The mRNAs corresponding to the two cDNAs were found to be coexpressed in equal amounts in all the B-CK-containing tissues tested so far (Perriard et al., 1987). We therefore adopted the working hypothesis that the two transcripts could have a common first exon and be under the control of a single promoter.

Such a putative exon 1 was found just upstream of the alternatively utilized H4-specific exon 2b. Its 3' end consists, as expected, of the 12-bp stretch present in the 5' portions of both cDNAs and in the 5' direction the 18c-derived 5'-terminal sequence follows (Fig. 1A, dark hatched box). This exon is followed by a canonical splice donor site, however, no splice acceptor could be detected at the location, where the homology between the genomic sequences and the cDNA 18c ended. Furthermore, about 50 bp further upstream, two promoter-like elements were found, each composed of a perfect TATA consensus sequence and a CCAAT box.

In order to test if these putative promoter elements are the ones used in vivo, we performed the S1 protection experiments and primer extension analysis shown in Fig. 3. Probe A (oligonucleotide 120589–46) spans the region from the postulated exon 1 (nucleotide number 32 of the cDNA sequence 18c) up to the distal TATA-like element (49 to 58 in Fig. 2) and is followed by 11 bp of extraneous sequence. This probe has been used in S1 protection experiments with different RNAs isolated from B-CK-containing tissues such as gizzard (Fig. 3A, lane 4), whereas RNA from adult skeletal muscle served as a negative control (Fig. 3A, lane 3). With gizzard RNA four major protected fragments of 58, 57, 56, and 51 bp length were seen. The same was true for RNAs from brain and heart (not shown) while no band occurred in the leg muscle RNA. The longest fragment extends up to a G nucleotide 22 bp 3' of the proximal TATA-box.

These results were corroborated by data obtained from primer extension analysis with two different synthetic oligonucleotides, one of them being H4-specific (Fig. 3B) and the other being directed against 18c-derived sequences, encoded mainly by exon 1 (Fig. 3C). The H4-specific probe B (oligonucleotide 940788–79) hybridizes to the transcripts in a region which is encoded by the alternatively used exon 2b (771–802, Fig. 2) and extends into exon 1 by 8 nucleotides (69–76, Fig. 2) i.e. the probe covers nucleotides 5–44 with respect to the cDNA sequence. Reverse transcription of gizzard poly(A)+ RNA gave rise to two products of 107 and 101 bp in length, respectively (Fig. 3B). Again, the same results were obtained with RNAs from brain and heart. The main extension product therefore maps to a G residue 1 bp further 3' than the longest protected fragment found in the S1 analysis. Thus, the values obtained by this primer extension can be considered to be in perfect agreement with the S1 data discussed above. The same is true for the primer extension performed with probe C (oligonucleotide 280688–75), corresponding to the cDNA 18c between nucleotides 5 and 54 (positions 30–76 and 1380–1385 in Fig. 2), which gave rise to two products of 78 and 72 nucleotides in length. The fact that only two products were found in the primer extension analysis is likely to be due to the lower resolution of the gels used in this assay. No transcripts corresponding to an activity of the distal B-CK promoter element were found with either S1 analysis or primer extension experiments at any developmental stage or in any B-CK expressing tissue tested.

According to our working hypothesis, the 5' end of the H4-type transcript must also contain the sequences found in the 5'-terminal part of the cDNA 18c and in exon 1 (dark hatched boxes in Figs. 1 and 3). To test this hypothesis, we performed another S1 analysis (Fig. 3D). The probe which was utilized spans part of the H4-specific sequence encoded by exon 2b (771–787 in Fig. 2, nucleotides 13–29 of the cDNA clone), the part of exon 1 (40–67, Fig. 2), which is contained within both cDNAs, as well as that which was only found in clone 18c (nucleotides 12–48 of the cDNA) and 11 bp of extraneous sequence at its 3' end. Analysis of brain total RNA revealed protection of the 18c-type sequences together with the labeled H4 end. A protected fragment of 53 bp length resulted, which indicates that exon 1 is indeed part of both transcripts derived from the B-CK gene. We therefore conclude that the proximal promoter-like element is the one which directs transcription of one type of hnRNA, and this RNA gives rise to two mature mRNAs by a non-regulated, alternative splicing mechanism.

Taken together, the data presented above suggest that the 5' most exon we have found is indeed exon 1 and that the proximal promoter-like element is the one which is active in vivo. This situation is reminiscent of that in the mammals, since there too, only the proximal of two promoter elements

Derivation from one B-CK locus in the chicken genome.
Fig. 2. Partial sequence of the chicken B-CK gene. The sequences were determined as indicated by the arrows in Fig. 1B. 5' flanking as well as 3' flanking sequences and introns are shown in lowercase letters, whereas exon sequences are in capital letters. The amino acid sequences are written in the single-letter code beneath the respective exon sequences. The numbering refers to the 5' most initiation site for transcription (termed +1) as determined by S1 nuclease protection analysis (black dots) as well as by primer extensions (vertical lines). The imperfect inverted repeat flanking the H4-specific exon 2b as well as putative regulatory sequences such as TATA-boxes, CCAAT-motifs, the polyadenylation signal (AATAAA), and the potential Sp1-binding sites (CCGCCC) are underlined. Portions of introns which have not been sequenced are indicated with dotted lines, and the length of the lacking sequence information is indicated in brackets.
is used (Benfield et al., 1988; Daouk et al., 1988; Mariman et al., 1987). Furthermore, a sequence motif ranging from position -49 to -83 in the chicken was found to be highly conserved among the different published B-CK promoters (Fig. 6, see "Discussion"). This evidence confirms that we have identified the B-CK gene promoter, although its activity remains to be investigated by direct promoter assays.

The B-CK Gene Is a Single Copy Gene—In order to determine the B-CK gene copy number, genomic Southern blot analysis has been performed, using high molecular weight genomic DNA from erythrocytes of two chickens of distinct White Leghorn inbred strains. This would enable us to detect different allelic variants or multiple genes/haploid genome.

The 2.5-kb HindIII fragment derived from the 3' end of the gene (Fig. 1B) was used as a probe and gave rise to only one hybridization signal/lane on Southern blots, when genomic DNA from the inbred strain 2883 digested with HindIII, TaqI, and KpnI was tested (Fig. 4A). The three fragments thus obtained were 2.5, 8.9, and 10.5 kb long, respectively. These data are in perfect agreement with the restriction map shown in Fig. 1B and therefore indicate that the B-CK gene in chicken is a single copy gene. The same results were obtained using DNA from the other inbred strain 2854 (not shown).

Furthermore, the cDNA probe H4 gave rise to one strong hybridization signal/lane on Southern blots, when genomic DNA from erythrocytes of two chickens of distinct White Leghorn inbred strains. This would enable us to detect different allelic variants or multiple genes/haploid genome.

The Two B-CK Transcripts Give Rise to Two Protein Subspecies, Ba- and Bb-CK, with Distinct N Termini—At the protein level, two isoproteins differing in their isoelectric points could be resolved by two-dimensional gel electrophoresis (Rosenberg et al., 1981), and these are now termed Ba- and Bb-CK. It has been shown by cell-free translation of in vitro generated RNA and subsequent comigration of the synthetic product with purified B-CK protein fractions on two-dimensional gels, that the more basic protein species, Bb-CK, is encoded by a transcript corresponding to the cDNA H4 (Perriard et al., 1987; Soldati et al., 1990).

We assumed that the Ba-CK isoform is encoded by a 18c-type transcript since the two protein subspecies as well as the two RNAs are coexpressed in similar amounts in all B-CK-positive tissues tested so far (Perriard et al., 1987). Unfortunately, the cDNA clone 18c lacks the C-terminal half of the protein coding sequence and, in addition, a screening of an Agt11 library for a full-length 18c cDNA was not successful. However, the structure of the gene that encodes the two transcripts strongly suggests that the 3'-terminal part of the two RNAs are identical, i.e. that the 3'-terminal part of the cDNA H4 (white hatched bar in Fig. 1) is also part of the 18c transcript. We therefore constructed a chimeric cDNA clone TW18-1, which contains the 5' end of the cDNA 18c, fused to the 3'-terminal part of H4 at the BamHI site at nucleotide 39b with respect to the H4 sequence (Fig. 1A). This construct was utilized for run-off transcription with the SP6 RNA polymerase and the resulting RNA served as a template for in vitro translation in the reticulocyte lysate system. The radioactive protein obtained by this procedure (Fig. 5d) comigrated on two-dimensional gels exactly with the Ba-CK species (Fig. 5d), a minor species was identified as the phosphorylation product of a truncated Bb-CK species, which arises by initiation of translation at the methionine 12 codon (Soldati et al., 1990). The fact that such a highly related protein is sorted out from the Ba-CK spot, while the synthetic protein derived from TW18-1 was dispersed throughout the whole Ba-CK spot (Fig. 5d), a minor protein species was excluded from the area occupied by Ba-CK (Fig. 5c, asterisk). This minor species was identified as the phosphorylation product of a truncated Bb-CK species, which arises by initiation of translation at the methionine 12 codon (Soldati et al., 1990).
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Figure 3. Mapping of the transcriptional start sites of the two mRNAs derived from the chicken B-CK gene. The probes used for the S1 analysis (A and D), the H4-specific primer extension (B), and the 18c-specific primer extension (C) are sketched in the graph at the bottom. Their lengths (in brackets) as well as their locations with respect to the 5' most transcription start site (+1) of the gene as determined by this analysis are shown (+30, +58, +69). The dotted arrows indicate the stretches which have been polymerized during the reverse transcription (B and C). The asterisks signify the radiolabeled ends of the probes. The two putative promoter elements (TATA) and the three 5' most exons (E1, E2b, E2a) are indicated. The black box represents 18c-specific sequences, the white one stands for H4-specific sequences, the dotted box indicates a sequence that is identical in both cDNAs and the dark hatched box represents the 5'-terminal sequence from the cDNA 18c (see Fig. 1A), while the cross-hatched part is not contained in any of the two cDNA clones. A, S1 analysis with probe A which encompasses the putative start site for transcription and contains 11 bp of extraneous sequence added at its 3' end (oligonucleotide 120189-46). 20 μg of total RNA from leg muscle (lane 3) and gizzard (lane 4) were hybridized at 55°C overnight to 5'-32P-labeled probe A (4.8·10^6 cpm/pm), digested with 30 units of S1 nuclease during 1 h, run on a 10% acrylamide gel under denaturing conditions and exposed to x-ray films for 16 h. Lane 1, pBR322 cut with HpaII; lane 2, undigested probe. H, H4-specific primer extension. 32P-labeled probe B (primer 040588-79, 5.2·10^6 cpm/pm) was hybridized to 2 μg of poly(A)+ RNA from gizzard (lane 1) and from leg muscle (lane 2) at 50°C for 2 h and elongated with Moloney murine leukemia virus reverse transcriptase in the presence of nonradioactive dNTPs at 37°C for 1 h. The products were separated on a 10% acrylamide gel under denaturing conditions and exposed to x-ray films for 18 h. C, 18c-specific primer extension. The labeled probe C (primer 280688-75, 5'·10^6 cpm/pm) was hybridized to 2 μg of poly(A)+ RNA from gizzard (lane 1) and from leg muscle (lane 2) at 60°C for 2 h and otherwise processed in the same way as probe B. D, S1 analysis with the chimeric probe D containing 18c-derived sequences from exon 1 as well as H4-specific sequences from exon 2b. Probe D (oligonucleotide 090589-40, 4.7·10^6 cpm/pm) was hybridized overnight at 48°C to 20 μg of total RNA from leg muscle (lane 2) and gizzard (lane 3), respectively, digested at 46°C for 1 h with 50 units of S1 nuclease, and run on a 10% denaturing acrylamide gel, which was exposed to a x-ray film for 15 h. Marker: pBR322 cut with HpaII; lane 1, undigested probe.

To further confirm that the in vivo produced Ba-CK mRNA has the same coding capacity as the TW18-1-derived transcript, and that the two are also equal at the nucleotide level, we performed hybrid-arrested translations in the reticulocyte lysate (not shown). Poly(A)+ RNA from brain, which was known to give rise to both species Ba- and Bb-CK after in vitro translation, was incubated with synthetic minus strand oligonucleotides complementary to 18c- and H4-specific sequences corresponding to the alternatively utilized exons, as well as with one primer complementary to a sequence stretch around position 950 in the part shared in the 3'-terminal portions of the cDNA clones H4 and TW18-1 (white hatched portion in Fig. 1). While inclusion into the assay of the H4- and 18c-specific primers led to a specific inhibition of Bb-CK and Ba-CK synthesis, respectively, the 3' specific primer abolished the translation of both B-CK subtypes. These results support further that the 3' portion of H4 codes for the C termini of Ba- as well as Bb-CK, and that there is no additional Ba-CK encoding transcript in brain RNA. Thus, we conclude that the chimeric cDNA clone TW18-1 very likely represents the Ba-CK mRNA sequence. Finally, the complete pattern of protein spots on two-dimensional gels derived from purified B-CK protein or from immunoprecipitated in vitro translation products of poly(A)+...
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**Fig. 4. Genomic Southern blots of DNA from two White Leghorn chicken inbred strains.** A, 4 µg of genomic DNA (from the strain 2883) per lane, digested with HindIII (lane 1), TstI (lane 2), and KpnI (lane 3) were transferred onto a nylon membrane and hybridized to the 32P-labeled 2.3-kb HindIII fragment (1.9-106 cpm/pm) that contains the 3' end of the gene (Fig. 1B). The blot was exposed to an x-ray film for 80 h. B, 4 µg of DNA from the strains 2854 (lanes 1 and 2) and 2883 (lanes 3 and 4), both cut with HindIII (lanes 1 and 3) or with EcoRI (lanes 2 and 4) and transferred onto a nylon membrane were hybridized with 32P-labeled cDNA of clone H4 (3.2-106 cpm/pm) and exposed to an x-ray film for 18 h. The HindIII fragments showing a putative restriction fragment length polymorphism are marked with arrowheads. Arrow indicates the positions of the HindIII fragments that were obtained by cloning polymerase chain reaction-amplified DNA. The markers (λ cut with HindIII and EcoRI) are indicated in kb.

RNA from brain (Fig. 5a) can be reconstituted by comigration of the in vitro generated protein products derived from the synthetic H4- and TW18-1 transcripts (Fig. 5c). This indicates that no additional RNA species for other isoforms of B-CK are present in chicken.

**DISCUSSION**

The chicken B-CK gene is the first CK gene characterized to date which codes for two proteins. An alternative splicing mechanism gives rise to two transcripts which encode two B-CK subspecies, Ba- and Bb-CK, with distinct N termini. Sequences containing an 18-bp, homologous T motif and a 17-bp homo-T sequence, respectively (nucleotides 584-625 and 1167-1234 in Fig. 2), are located on either side of exon 2b, forming an inverted repeat. This motif might fold into a stem-loop structure, containing the alternatively utilized exon in the loop. A computer analysis (Zuker, 1981), in which 100 bp flanking the region of interest have been included, confirmed the postulated potential secondary structure. The free enthalpy of such a structure was calculated to be -18.6 kcal/m (Tinoco et al., 1973). It is tempting to speculate that this sequence motif, even if giving rise to a secondary structure of only "middle" stability, is somehow involved in the differential splicing mechanism.

The transcription of the two mRNAs is directed by a complex promoter region, which shows a high degree of homology to the promoter regions of the mammalian B-CK genes (Fig. 6). All the B-CK genes, including that of the chicken, contain two promoter-like elements within their 5' flanking regions. The proximal elements direct the transcription of all the B-CK RNAs whereas the distal elements have been reported to be silent. In the chicken each of these two elements consists of a perfect TATA consensus sequence and a CCAAT-box, while in the mammalian systems the respective sequences of the two elements differ considerably. There, the proximal elements contain a "TATA" motif which has been shown to be the active "TATA" box (Benfield et al., 1988; Daouk et al., 1988; Mariman et al., 1987) and the distal element contains a canonical TATA box sequence. These two sequences can be discriminated by a DNA-binding activity described in the rat, i.e. the TA-rich DNA-binding protein.

**Fig. 5. Reconstitution of the B-CK protein pattern on two-dimensional gels derived from poly(A)+ RNA by in vitro translation of synthetic RNAs.** The 5' end of the cDNA 18c and the 3' end of the gene (see Fig. 1A). This construct was linearized with HindIII and used for run off transcription with the SP6 RNA polymerase. Panels a-d represent autoradiographs of two-dimensional gels on which [35S]methionine-labeled in vitro translation products derived from different RNA templates were resolved. Arrowheads point to the B-CK isoproteins Ba- and Bb-CK as seen after staining the blots for protein (not shown). All translations have been carried out in the reticulocyte lysate system. A, immunoprecipitated translation products from 3 µg of brain poly(A)+ RNA; B, translation product from synthetic H4 RNAs; C, translation product from H4-derived transcripts comigrated with purified B-CK from brain. The asterisk indicates the phosphorylation product of a Bb-CK subform generated by initiation of translation at methionine 12 (Soldati et al., 1980). D, translation product from TW18-1-derived RNA comigrated with purified B-CK from brain.

These findings are somewhat surprising since, in general, chicken genes do not appear to be longer than their mammalian analogues.

A striking sequence feature has been identified flanking the H4-specific alternatively spliced exon 2b. Two very AT-rich sequences, containing a 17 bp long homo-T and a 17-bp homo-A stretch, respectively (nucleotides 584-625 and 1167-1234 in Fig. 2), are located on either side of exon 2b, forming an inverted repeat. This motif might fold into a stem-loop structure, containing the alternatively utilized exon in the loop. A computer analysis (Zuker, 1981), in which 100 bp flanking the region of interest have been included, confirmed the postulated potential secondary structure. The free enthalpy of such a structure was calculated to be -18.6 kcal/m (Tinoco et al., 1973). It is tempting to speculate that this sequence motif, even if giving rise to a secondary structure of only "middle" stability, is somehow involved in the differential splicing mechanism.

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3 U. Achtnich, personal communication.
Figure 6. A comparison of the B-CK promoter sequences from human (Mariman et al., 1987) (a), rat (Benfield et al., 1988) (b), and chicken (c) is shown. Both the human (a) and the chicken promoter (c) are compared with that of the rat (b) and sequence identity is indicated by vertical lines. Gaps introduced for best alignment of homology are marked by dots. TATA and CCAAT boxes as well as CACCC motifs and potential SP1-binding sites (GGGCGG and CCGCCC) are underlined. The black bar below the alignment spans the most rigorously conserved sequences. The variability of the more proximal sequences containing the active TATA boxes is clearly much higher. The two TATA boxes in the chicken B-CK promoter. However, a comparison of the 5' flanking region of the chicken gene to those of the mammalian genes revealed that the region containing the TARP-binding site as well as the two CCAAT boxes is by far the most rigorously conserved part of the B-CK promoters. Within this stretch, which reaches from position -48 to -83 in the chicken, only two mismatches occur between the chicken and the mammalian sequences. The variability of the more proximal sequences containing the active TATA boxes is clearly much higher. It has been shown by footprinting studies and gel retardation assays as well as by competition experiments using synthetic oligonucleotides spanning parts of the conserved region, that the TARP binding depends also on some nucleotides flanking the distal TATA box. On this basis a TARP-like molecule might also be able to discriminate between the two TATA boxes in the chicken B-CK promoter. However, a high selective pressure must have been responsible for the conservation of the TARP-binding region, indicating that the TARP (or an analogous activity in chicken) fulfills an important, as yet undefined, task in the regulation of the B-CK genes. Finally, it may be important to note that the spacings of the different putative cis-active elements in the promoter sequences, which are compared with each other in Fig. 6, are very well conserved except for the potential SP1-binding sites, which differ in number, orientation, and location between the different species.

At the protein level, a comparative analysis of all the CK primary structures known to date (B-, M-, and Mi-CKs) revealed that there are several stretches of amino acid sequence identity, which are dispersed throughout the whole protein coding region (Babitt et al., 1986; Hassle et al., 1988). These stretches constitute a "CK framework," which is believed to be important for enzymatic activity and other "general" CK-specific tasks (Hassle et al., 1988). Furthermore, some residues that are conserved in cytosolic forms (M- and B-CK versus Mi-CK) and some which appear to be specific for certain isoforms have been identified.

The most obvious difference between the mammalian B-CKs characterized so far and the chicken B-CK isoproteins is the existence of the two subspecies Ba- and Bb-CK in chicken versus the single B-type isoforms in the mammals (see also Figs. 7 and 8). In Bb-CK the translation start site at the first methionine codon of the transcript 3' of the cap structure has been confirmed by direct protein sequencing (Quest et al., 1990; Hassle et al., 1988) and it has been suggested that the translation start of Ba-CK is also at the first AUG of the respective transcript, since it is part of a good consensus sequence for translation start sites (Kozak, 1986). However, this could not be verified by Edman degradation of the protein due to a blocked N terminus (Quest et al., 1990). Thus, the resulting Ba-CK protein is predicted to be only 376 amino acids long, while Bb-CK as well as all other cytosolic CKs contain 381 residues (including Met-1). In addition, the Ba-CK protein does not contain the features in the N-terminal portion which were expected to be typical for cytosolic isoforms (Fig. 7). Histidine 7 and lysine 11, which are found in all cytosolic CKs characterized so far, are replaced by alanine and asparagine, respectively, in Ba-CK.
Furthermore, the isotype-specific residue 18 which is aspar- 
glu atate in all B CKs and glutamate in M CK, this glutamate in Ba-CK. This glutamate is part of a stretch of 3 glutamate 
residues in Ba-CK which is collinear with the respective part 
of the chicken M-CK sequence; further N terminally two 
proline residues follow, which are also found in the respective 
location in all Mi-CK proteins characterized. Therefore it 
looks as if the N-terminal part of Ba-CK is a "mosaic-CK," 
containing sequences from different CK isoforms.

These distinct N-terminal sequence features could be re- 
sponsible for some differences in the biophysical properties 
as well as in the biological behavior of the two B-CK subspe- 
cies. For example, it has been shown in our laboratory that 
additional minor B-CK protein subspecies are produced in 
vivo and can be resolved on two-dimensional gels. These 
minor subspecies can also be generated by in vitro translation 
of synthetic B-CK RNAs and were shown to be due to 
alternative initiation of translation (Soldati et al., 1990; see 
also Fig. 7). The analysis of mutant Bb-CK transcripts re- 
vealed that in Bb-CK the initiation of translation can take 
place at methionine 12, leucine 36, methionine 30, and me- 
thionine 70, in addition to methionine 1; the product of 
initiation at methionine 12 is the most prominent species 
generated by this mechanism. In vitro translation of synthetic 
Bb-CK RNA also gives rise to minor species comigrating 
with the products of initiation at methionine 30 and the two 
downstream sites (not shown). However, the product of ini- 
tiation at methionine 12 is absent since no methionine is 
present in the N-terminal part of Ba-CK. The fact that these 
minor species also occur in vivo suggests that they may play 
a physiological role and, therefore, the potential of the Bb- 
CK transcript to give rise to the product of initiation at 
methionine 12 must be regarded as an important difference 
in coding capacity compared with the Ba-CK RNA.

In the rat (Mahadevan et al., 1984), it has been shown that 
B-CKs are phosphoproteins, and the same could be shown for 
the Bb-CK isofrom in chicken.4 No conclusive data are yet 
available on whether Ba-CK is also phosphorylated, and, if it 
is, whether there are differences in the phosphorylation pat- 
tterns of the two subspecies. In this respect it should be noted 
that Bb-CK contains 4 serines more than Ba-CK due to 
differences in their N-terminal parts (Fig. 7). It remains to be 
shown whether one or the other of these residues is involved 
in the phosphorylation; however, this question is of great 
importance, since there is good evidence that the enzymatic 
activity of B-CK is modulated by phosphorylation.

As mentioned above, Ba-CK appears to have a blocke N 
termis.5 Neither the structure nor the function of this block 
is known to date, but it is clear that this modification is Ba- 
CK-specific. Therefore, we can assume that the sequence 
information which is needed for this modification must also 
reside within the very N-terminal portion of Ba-CK.

The exceptional heterogeneity of the B-CK proteins in 
chicken might account for the observation that the chicken 
expresses B-CKs, but no M-CK, in the adult heart, while the 
mammals coexpress D- and M-CK. Furthermore, neither Mi- 
CK protein nor its activity could be detected in chicken 
gizzard at any developmental stage in spite of the presence 
of B-CK. It is likely that the B-CKs therefore have to fulfill 
additional tasks in these cellular backgrounds, as compared 
with those in which further isoforms are coexpressed with 
the B-type protein. In addition to this, it has been shown recently 
that the heterodimer formation of the two B-CK isoforms is 
regulated in neuronal tissues but not in muscle cells.4 These 
findings support the idea that the three B-CK isoenzymes 
(BaAs-, BaBb-, and BbBb-CK) play distinct, as yet undefined, 
physiological roles. With respect to the CP shuttle model 
(Bessman and Geiger, 1981; Wallimann and Eppenberger, 
1985; Wallimann et al., 1989) it is tempting to speculate that 
the N-terminal differences between the two B-CK subspecies 
could lead to differential association with distinct cellular 
structures involved in high energy turnover.

The chicken is so far the only vertebrate which has been 
reported to express two B-CK protein subspecies. However, 
in the case of the human B-CK it has been shown that in 
addition to the active gene on chromosome 14, another 
B-CK-like gene is located on chromosome 16 (Kaye et al., 1987; 
Ma et al., 1988); it has not yet been demonstrated whether 
this additional gene is active or not. It would be important to 
know whether the chicken is an exception with respect to the 
B-CK heterogeneity or whether we are dealing with a general 
avian-specific feature. An analysis of the genetic material of 
other avian species for the presence of Ba- and Bb-CK-like 
sequences should shed more light on this question.

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