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Functional identification of the promoter for the gene encoding the α subunit of calcium/calmodulin-dependent protein kinase II

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ABSTRACT **To examine the expression of the α subunit of calcium/calmodulin-dependent protein kinase II, various 5′ flanking genomic sequences were inserted into a chloramphenicol acetyltransferase (CAT) reporter plasmid and CAT enzyme activities were analyzed in transfected NB2a neuroblastoma cells and mRNA transcription was analyzed by nuclease protection assays. A core promoter was identified which contained an essential TATA element located 162 nt 5′ to the transcription start site. Sequences 3′ to the transcription start site, as well as 5′ to the TATA element, increased levels of CAT activity in transfected cells. The α-subunit gene promoter displayed higher CAT activities, relative to a simian virus 40 promoter, in transfected neuronal cell lines than in nonneuronal cell lines. Results also suggested that sequence surrounding the natural α-gene transcription initiation site may be important for targeting transcription initiation 162 nt downstream of its TATA element.**

Dramatic differences are observed in the expression of the α subunit of calcium/calmodulin-dependent protein kinase II (CK2) and its mRNA during brain development (1, 2) and in different adult brain regions (3). The CK2 α-subunit mRNA and protein increase markedly in postnatal brain during the most active phase of synapse formation. In contrast, the β subunit is readily detected at birth and increases little during postnatal development. CK2 is one of the most abundant protein kinases found in mammalian brain and constitutes ≈1% of the total protein in the hippocampus (3), where its mRNA levels are also high (2), whereas the α-subunit mRNA is very low in cerebellar granule cells and Purkinje neurons (2).

CK2 plays an important role in events that underlie the induction of long-term potentiation (4, 5), and some have speculated that such synaptic plasticity may involve increases in the transcription of CK2 mRNA (6). Recent studies using transgenic mice further indicate that CK2 α subunit may play a role in spatial learning (7). The developmental and neuron type-specific expression of CK2 α mRNA and α-subunit protein (2, 8–12) indicates that α-gene expression is carefully regulated at the level of transcription. The α-gene 5′ flanking region was previously examined (13), but promoter activity was not investigated. We have studied α-gene expression by examining genomic sequences that are necessary and sufficient for α-gene promoter activity. We identified a core promoter with an unusually positioned but necessary TATA element. Evidence is presented regarding the importance of the initiation sequence in α-gene transcription and the identification of regulatory regions that affect α-gene promoter activity.*

MATERIALS AND METHODS

Isolation of Genomic Clones. A rat genomic library in λ phage Charon 4A (Clontech) was screened by conventional Southern blot techniques using a 1650-nt CK2 α-subunit cDNA fragment containing the ATG translation start codon. Ten nonoverlapping genomic clones were isolated, and selected regions encompassing the 5′ flanking sequence and the CK2 α coding region were subcloned into pGEM-3Zf(−) (Promega) and sequenced by the dideoxy method (14).

Plasmid Construction and Transfections. CK2 α-gene constructs I–V were prepared by inserting genomic DNA fragments from the 5′ flanking sequence of the α-gene into pCAT Enhancer (see Fig. 2). This reporter plasmid (Promega) contains the chloramphenicol acetyltransferase (CAT) coding region plus the simian virus 40 (SV40) enhancer but contains no promoter. NB2a neuroblastoma cells (ref. 15; from Tom Shea, Harvard Medical School) were cotransfected with 15 μg of CsCl-purified plasmid DNAs, including 1 μg of pRSV-β-gal, by the calcium phosphate method (16). Cells were harvested after 40 hr, and cell extracts and/or total cellular RNA were prepared as described (17).

RNase Protection Assays. Transcription start sites for α-gene constructs I–V were determined by RNase protection assays using RNA probes derived from pGEM-3Zf(−) containing subcloned inserts composed of both α genomic and pCAT Enhancer sequences (subcloned from corresponding α-gene constructs) (see Fig. 2). Total cellular RNA fractions (12 μg) from cells transfected with pCAT Enhancer or the α-gene constructs were denatured and hybridized at 60°C to ^{32}P-labeled single-stranded antisense RNA probes (5×10^5 cpm) and subsequently digested with RNase T_1 (3000 units/ml) plus RNase A (40 μg/ml) for 60 min at room temperature. Protected ^{32}P-labeled RNA fragments were sized by electrophoresis in denaturing polyacrylamide gels with dideoxy sequencing ladders as calibration standards.

Protection assays using RNAs from cells transfected with uncut pCAT Enhancer revealed a major protected cRNA fragment of 275 nt (data not shown). The generation of this protected transcript from the promoterless pCAT Enhancer indicated that a cryptic promoter existed within or 5′ to its cloning cassette and, thus, 5′ to the α-gene sequences in the different constructs (see Fig. 2). This was confirmed by the generation of larger-than-predicted cRNA fragments in some assays. For example, hybridization of RNA probe I to RNA from cells transfected with uncut construct I produced a 570-nt cRNA fragment (see Fig. 3, lane 3), equal in length to the entire α-gene insert (295 nt) plus pCAT sequence (275 nt) (see Fig. 2). Thus, transcription clearly initiated 5′ to the α-gene insert in construct I. A successful strategy was designed that virtually eliminated cryptic promoter activity by cutting immediately 5′ to the α-gene sequences in the different reporter constructs prior to transfection (see Fig. 3, lane 2). Despite the generation of mRNAs by the cryptic promoter in cells transfected with uncut plasmids, little if any detectable CAT activity was associated with cryptic transcripts (see Fig. 4 and below).

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Abbreviations: CK2, calcium/calmodulin-dependent protein kinase II; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

*The upstream sequence of the CK2 α gene has been deposited in the GenBank data base (accession no. M29699).

CAT Assays. Cell extracts (25 μl) were used in a CAT diffusion assay (18) which measures the kinetics of [^{3}H]acetylated chloramphenicol formation. Kinetic assays for β-galactosidase activity were conducted and activity ratios (CAT activity/β-galactosidase activity) were determined by using β-galactosidase as an internal control for transfection efficiencies. Normalized CAT activities in extracts from transfections with cut vs. uncut α-gene constructs were very similar (see Fig. 4). CAT activities observed with the various constructs were produced predominantly by α-gene promoter-driven transcripts with little or no contribution by cryptic promoter activity (discussed above). Uncut constructs IV and V produced exclusively cryptic promoter RNA (data not shown) but generated no CAT activity (see Fig. 4*B*). The absence of CAT activity in construct V is most likely due to two in-frame ATG translation start codons between −61 and −3 (9 bp apart), followed 21 bp downstream by an in-frame stop codon, whereas construct IV contains an ATG codon (regenerated during subcloning) just 53 bp upstream but out of frame with the CAT gene ATG codon (see Fig. 2). If the scanning theory in which ribosomes preferentially begin translation at the first ATG holds true (19–21), then any translation from these upstream codons in the cryptic promoter transcripts would produce nonfunctional protein and might simultaneously interfere with efficient translation initiation at the downstream CAT gene ATG (e.g., see uncut constructs V and IV in Fig. 4*B*). In contrast, uncut constructs I and II, while subject to the same restraints in translation of their cryptic RNAs, also produced large amounts of α-gene transcripts which initiated 3′ to the −61/−3 region (see Fig. 2) and therefore did not contain the upstream ATG codons found in the larger cryptic transcripts. These α-gene transcripts begin translation exclusively at the CAT gene ATG and produce substantial CAT activity (see Fig. 4).

RESULTS

Isolation and Characterization of CK2 α-Subunit Gene. α-Gene clones were isolated containing ≈13 kb of 5′ noncoding sequence; 2.5 kb of genomic sequence 5′ to the translation ATG start codon was sequenced. The mRNA encoding the CK2 α subunit is 4852 nt long and contains a large 3′ noncoding sequence, 3271 nt (Fig. 1*A*). Seven exons were identified which encompassed ≈27% of the deduced cDNA sequence (Fig. 1*B*). The first exon contains 147 nt of 5′ noncoding sequence and encodes the N-terminal 21 aa of the α-subunit (Fig. 1*B*). Six additional exons were identified and five of their intron/exon boundaries conformed to the NYAG[G . . . YAG]GT rule (22).

The mRNA transcription start site was determined by primer extension sequencing using two oligonucleotides (Fig. 2*A*, oligonucleotides b and c). Sequence analysis verified that

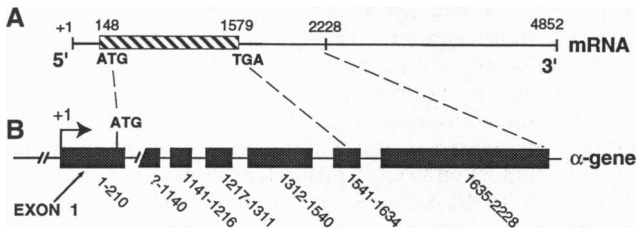

FIG. 1. Spatial relationships between the full-length CK2 α mRNA (*A*) and the α gene (*B*). (*A*) CK2 α mRNA coding region (hatched rectangle) and 5′ and 3′ untranslated regions; translation initiation and termination codons begin at positions 148 and 1579, respectively. (*B*) Exon/intron relationships in the α gene; solid rectangles indicate exons (numbers below each exon designate the corresponding nucleotide sequence in the mRNA); ATG defines the translation start codon, and the arrow denotes the transcription initiation site at nt +1.

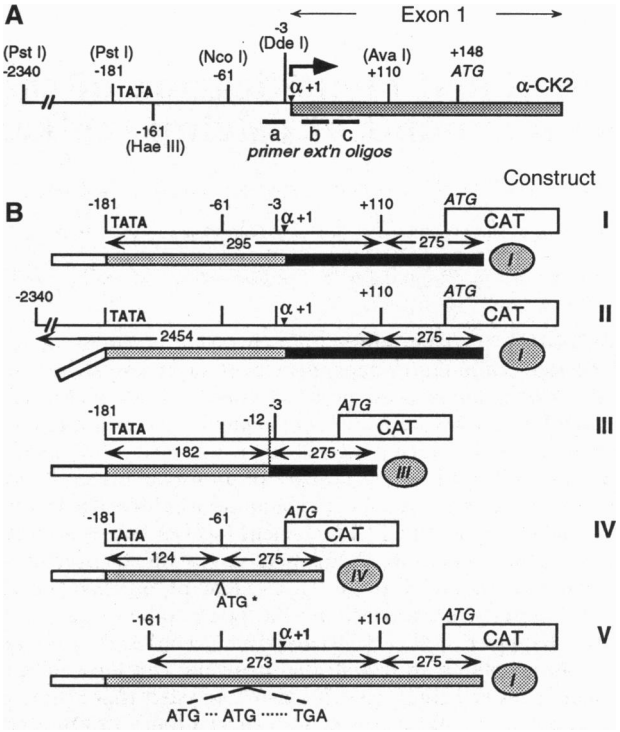

FIG. 2. Organization of α-gene promoter (*A*) and reporter constructs and RNA probes used in RNase protection assays (*B*). (*A*) Expanded view of the α gene showing exon 1, the transcription initiation site (+1), and core promoter region (restriction sites used in subcloning are indicated); oligonucleotides used in primer extensions are indicated (a, b, and c), along with the TATA element. (*B*) The α-gene inserts and pCAT sequences in each reporter construct are denoted by numbers (base pairs) within the double arrows. α-Gene sequences were subcloned into the pCAT Enhancer plasmid; CAT coding regions are indicated by white boxes. Fragments from the various constructs were subcloned into pGEM-3Zf(−) to generate antisense RNA probes I, III, and IV (stippled ovals). α-Gene, pCAT Enhancer, and pGEM (white bar) sequences in each RNA probe are indicated by horizontal bars under each construct; black regions represent protected cRNAs observed in RNase protection assays (see Fig. 3). The locations of two ATG start codons and a TGA stop codon (all in frame) that are present in constructs I, II, III, and V are indicated in construct V, and the location of an ATG codon (starred) generated during subcloning at the *Nco* I restriction site is indicated in construct IV.

RNA synthesized from each oligomer was complementary to the α-gene sense strand and terminated 147 nt 5′ to the translation initiation codon ATG (data not shown), confirming previous results from S1 nuclease protection assays (13). An oligonucleotide primer located directly 5′ to the transcription start site (Fig. 2*A*, oligonucleotide a) failed to initiate detectable extension products.

α-Gene 5′ Flanking Sequences Used in Reporter Constructs. A solitary consensus TATATAA sequence was identified 162 bp 5′ to the α-gene transcription start site. There are no TATA or CCAAT sequences in standard positions 5′ to the transcription start site (23). Because of the unusually long distance between the TATA element and the site of transcription initiation, it was important to establish whether this region of the α gene contained promoter activity. Plasmids were constructed with α-gene promoter sequences inserted into a pCAT Enhancer reporter plasmid (Fig. 2*B*). Construct I (−181/+110) contained the TATATAA and α-gene transcription start site. Construct II (−2340/+110) included the sequence in construct I plus 2159 bp of 5′ sequence (Fig. 2). Construct III (−181/−3) lacked the 113-bp region (−3/+110) in construct I, including the natural α-gene transcription start site. Construct IV (−181/−61) lacked the sequence −61/−3

present in construct III. Construct V (−161/+110) was similar to construct I, except that the TATATAA element (−181/−161) was absent.

Analysis of Functional α-Gene Promoter by RNase Protection Assays. To identify the α-gene promoter, it was important to determine whether (*i*) a specific α-gene region can promote mRNA transcription, (*ii*) transcription initiated in α-gene constructs is at the same site that naturally occurs in genomic DNA, (*iii*) sequences known to be important for gene transcription (e.g., TATA and initiator sequences) are necessary for α-gene expression, and (*iv*) the expression of mRNA in cells transfected with the various reporter constructs correlates with measurements of CAT activity.

RNase protection assays with RNAs synthesized in transfected cells were used to compare the sizes of different transcription products with predicted sizes based on the natural transcription start site of α-mRNA in rat hippocampus. Experiments in which RNA probe I was hybridized to hippocampal mRNA and subjected to RNase digestion resulted in a prominent 109-nt protected fragment and thus confirmed the natural α-gene transcription start site at 162 nt 3′ to the TATATAA sequence (data not shown).

Identification of the α-Gene Promoter. If α-gene promoter-driven transcription in transfected cells is similar to brain, then transcription should initiate 162 bp 3′ to the TATATAA (i.e., position +1; see Fig. 2). Constructs I and II (Fig. 3, lanes 2 and 4), which maintained the spatial relationship between the TATA and the natural α-gene transcription start site, demonstrated that α-gene transcription faithfully initiated at position +1. Both constructs I and II produced a major protected cRNA fragment of 384 nt which equals the 109 nt of α-gene sequence located 3′ to its transcription start site plus 275 nt of pCAT sequence (Fig. 2). In addition to transcripts initiated at the natural start site, a smaller protected fragment of 351 nt was observed. This possibly resulted from transcription initiated at position +34 in a pyrimidine-rich sequence which closely resembles a eukaryotic initiator sequence (24) or may simply represent a degradation product of the larger α-gene transcript.

Protection assays using RNA probe III and construct III, which retained the TATA element but not the natural α-gene transcription start site, produced a predominant cRNA fragment of 287 nt (Fig. 3, lane 8). In some experiments a much fainter 275-nt fragment was observed. These results indicated that transcription initiated primarily 12 nt upstream of the natural α-gene start site when the natural site was absent (Fig. 2*B*, construct III). Sequence analysis revealed that this prominent transcription start site at −12 in construct III was surrounded by a sequence that closely resembled the natural α-gene initiation sequence (see *Discussion*).

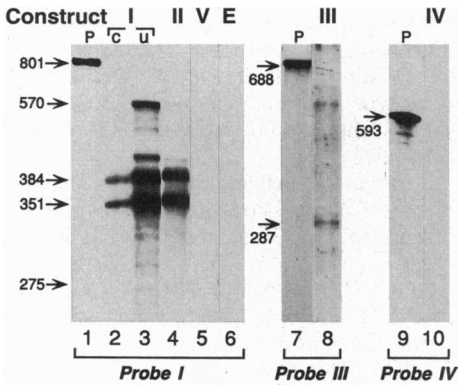

FIG. 3. RNase protection analysis. Antisense cRNAs (probes I, III, and IV) were hybridized to total RNA from cells transfected with cut constructs I–V (see Fig. 2) and pCAT Enhancer (E). Lanes 2 and 3 show protected RNA from transfections with cut (c) and uncut (u) construct I. Three gels are shown and the sizes (nt) of RNA probes (P; lanes 1, 7, and 9) and protected cRNAs are indicated. RNA probes were synthesized from pGEM-3Zf(−) and were always larger than their homologous sequence in corresponding RNAs generated during transfections.

Contributions of TATA and Other Sequences to α-Gene Promoter Activity. Hybridization of RNA from transfections with the TATA-less construct V to RNA probe I resulted in no protected cRNAs (Fig. 3, lane 5), demonstrating that the TATA element is required for α-gene promoter expression. It was important to determine what additional sequences might be required for promoter activity. No protected cRNA fragments were detected following transfections with construct IV (lane 10), which contains the TATA element but little additional 3′ sequence. The α-gene sequence in construct IV is identical to the fragment previously reported to display retarded migration in gel shift assays when pre-incubated with rat brain nuclear extracts (13). Our results show that this fragment (−181/−61) is not sufficient to initiate transcription, and sequences between −61 and −3 (construct III) are necessary for α-gene core promoter activity. Thus, two different regions were identified that are critical for α-gene core promoter activity, namely the TATA element and sequences between −61 and −3.

CAT Activity Measurements Confirm the Identity of the α-Gene Promoter. Our analysis of mRNA transcription identified the α-gene core promoter. However, analyses of CAT activities (i.e., the resulting translation product) in transfected cells provided additional important information regarding α-gene promoter function. Construct I consistently produced high levels of CAT activity and was used to normalize CAT activities determined for all plasmids (Fig. 4). pCAT Control, which contains both the SV40 promoter and the SV40 enhancer, produced 34% of the CAT activity of construct I (Fig.

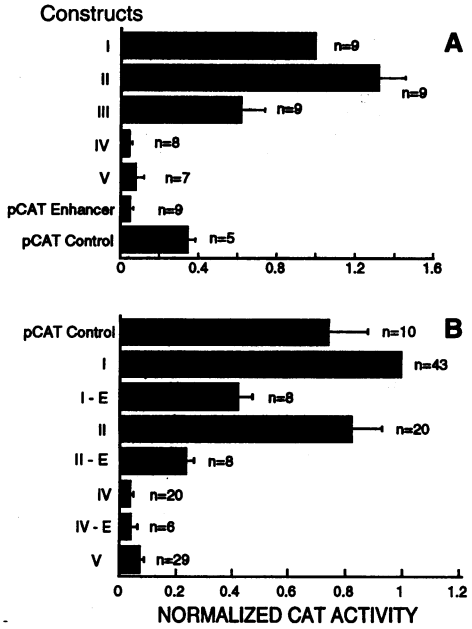

FIG. 4. Normalized CAT activities in NB2a cells transfected with various α-gene promoter constructs (*A*) or constructs lacking the SV40 enhancer (*B*). Values within experimental groups were averaged and normalized relative to the activity of construct I (error bars indicate SEM). (*A*) All plasmids were cut in the pCAT Enhancer cloning cassette at the *Pst* I site (or *Hin*dIII in construct II to avoid restriction at the internal *Pst* I site at −181) plus two additional *Pvu* II sites to prevent religation. (*B*) The SV40 enhancer was removed from certain constructs (−E) by cutting at *Nar* I and *Hpa* I sites and religating; uncut constructs containing enhancers and the pCAT Control plasmid were also used.

4*A*). In contrast, CAT activity produced by pCAT Enhancer (which contains the SV40 enhancer and no promoter) was only 5% relative to construct I (Fig. 4*A*).

Analysis of CAT activities with the various α-gene constructs was consistent with RNase protection results. The TATA-less construct V and construct IV, which failed to produce detectable cRNAs, both exhibited very low CAT activities (7% and 4%, respectively) relative to construct I (Fig. 4*A*). In contrast, constructs I–III, which generated significant α-gene transcripts (Fig. 3, lanes 2, 4, and 8), all exhibited high CAT activities (Fig. 4*A*). Construct III produced CAT activities that were 62% of those produced by construct I, indicating that sequences between −3 and +110 increased CAT activity. CAT activity with construct II was 33% greater than that with construct I. The relative contributions of the two major α-gene transcripts from constructs I and II (Fig. 3, lanes 2 and 4) to the total measured CAT activity are unknown. Taken together, these results confirm that construct III contains the α-gene core promoter.

Effect of SV40 Enhancer. Removal of the SV40 enhancer from constructs I and II resulted in 58% and 76% decreases in CAT activities, respectively (Fig. 4*B*). CAT activities measured with enhancerless plasmids were normalized to CAT activities observed with the corresponding plasmids that contained the SV40 enhancer. The enhancerless construct IV exhibited virtually no change in CAT activity compared with its enhancer-containing counterpart. These results indicate that α-gene promoter activity, while stimulated by the SV40 enhancer, is not dependent on it for activity.

Promoter Activity in Nonneuronal Cell Lines. All analyses of α-gene promoter activity to this point were conducted with NB2a neuroblastoma cells. Because of the neuron-specific expression of the CK2 α-subunit gene (2, 25), we examined promoter activity in nonneuronal and additional neuronal cell lines. Transient transfections were used to compare the promoter activity of construct I with the SV40 promoter in pCAT Control. The latter was expected to display consistently high levels of activity in the various cell lines (26). Cut plasmids were used to eliminate cryptic promoter activity. Construct I consistently displayed greater CAT activities than pCAT Control in NS-20 (2-fold), NG-108 (2.5-fold), and NB2a (2-fold) cell lines. In contrast, the SV40 promoter produced greater CAT activities than construct I in the nonneuronal cell lines COS-1 (7-fold), COS-7 (2-fold), and HeLa (2-fold). Thus although the α-gene promoter was active in nonneuronal cells, its expression relative to the SV40 promoter was consistently greater in neuronal cell lines.

DISCUSSION

α-Gene Promoter Identified. The CK2 α-subunit gene contains no consensus TATA sequence in the standard position of −25 to −30, nor any CAAT sequence at −60 to −70 (23). The TATA at −162 in the α-gene core promoter was absolutely necessary for RNA transcription in NB2a cells. Reporter constructs containing this α-gene TATA element, the transcription start site, and additional 5′ flanking sequence initiated transcription at the same site as the natural α gene in brain (i.e., 162 bp 3′ to the TATA) and produced CAT activities that were 194% greater than those directed by the SV40 promoter. The TATA-less construct V, whose α-gene sequence begins just 1 bp 3′ to the TATA element, exhibited virtually no promoter activity. The long distance between this TATA element and its transcription start site is a clear example that TATA elements can function at considerable upstream distances. Other promoters contain TATA elements as far as 100 bp 5′ to their transcription start sites, although not all have been examined for functional promoter activity (27–30).

Potential Promoter Regulatory Sequences. The additional 113 bp of α-gene sequence in construct I relative to construct III resulted in a 62% increase in CAT activity. This suggests that the −3/+110 region, while not required for core promoter activity, increases promoter activity, perhaps by restoring the natural transcription initiation site or by providing other transcriptional and/or translational regulatory sequences. In this context, there are numerous examples of promoters with transcriptional (31–33) and translational (34) regulatory sequences located 3′ to the transcription start site.

Construct II exhibited 33% and 71% greater CAT activity than construct I and construct III, respectively, and displayed transcription initiation properties identical to those of construct I. Construct II contains all of the α-gene sequence in construct I (or construct III) plus 2159 bp of additional upstream sequence. A search for consensus regulatory sequences in construct II revealed two previously reported CAACGG presumptive v-Myb binding sites at −87, −148 (13), plus an additional site at −472 and GGGCG Sp1-like binding sites at −77 and −967. A β-interferon silencer-like sequence is present at −274, a $(GA)_{27}$ repeat at −626, a potential serum response element (SRE) at −1145, a consensus AP1 sequence (TGAGTCAG) at −1410, a cAMP-inducible (CRE-like) ATF recognition sequence at −1761, an octomer (Oct 1) that may bind NF-3, OTF-1, and NF-A1 at −1924, and an E_2 viral enhancer-like sequence at −2005 (see refs. 35 and 36).

Sequences 5′ to the Transcription Initiation Sequence. Experiments with construct IV demonstrated that removal of the −61/−3 sequence from the core promoter resulted in a complete loss of promoter activity, possibly due to loss of the transcription start site at +1 and a potential start site at −12. This region is G+C-rich (67%) and bordered by an upstream Sp1-like box, although the latter is generally considered more important in TATA-less promoters (37). The −61/−3 region also contains two ATG translation start codons followed by a stop codon, all in the same reading frame (Fig. 2). These sequences are not present in the α-gene transcript in brain, but they could conceivably block translation of CK2 α mRNA in nonneuronal tissues, perhaps in a manner similar to the ribosome scanning theory (19–21).

α-Gene Promoter Activity in Nonneuronal Cells. Construct I consistently exhibited 2- to 2.5-fold more CAT activity in neuronal cell lines when compared with pCAT Control (i.e., the SV40 promoter). In contrast, pCAT Control exhibited 2- to 6-fold greater CAT activity than construct I in nonneuronal cell lines. Even though construct I contains only 91 bp of α-gene sequence in addition to the α-gene core promoter, its higher CAT activities in neuronal cell lines compared with pCAT Control indicate that α-gene promoter activity is generally higher in neuronal than in nonneuronal cell lines. Perhaps the neuron-specific expression of the α-gene *in vivo* may be regulated by additional silencer-like elements, as demonstrated for the type II sodium channel (38), SCG10 (39), and synapsin I (40), that would prevent CK2 α expression in nonneural tissues.

α-Gene Initiation Sequences. Our results suggest that specific recognition sequences contribute to the precise initiation of α-gene transcription. Constructs I and II initiated at the natural α-gene transcription site +1 ('A') in the sequence CTC'A'GAAGCCC, as well as at +34 ('A') in the pyrimidine-rich sequence TTCTCC'A'TTTGC, which is homologous to the eukaryotic consensus transcription initiator sequence YYC'A'YYYYY (41). Mutational studies have identified functional initiator sequences that position and enhance transcription in promoters that lack TATA elements or that have TATA elements in standard positions (41, 42). The α-gene TATA element required for promoter activity appears to exhibit specificity in its selection of initiation sequences, at either the natural initiation sequence or the site at +34. Initiation at position +34 has not been detected in brain and may result from a more relaxed control of transcription in NB2a cells.

Construct III, designed to remove the natural α-gene transcription initiation site CTC'A'GAAGCCC and the potential initiation sequence at position +34, initiated transcription at position −12 in the homologous sequence CAC'A'GTGCCC (transcription initiated at the 'A' in both). It is not known whether either of the alternative initiation sites (+34 or −12) is unique to transcription in cultured cells or might serve as a functional initiation site in other tissues (e.g., nonneuronal). The sequence at position −12 is part of the core promoter, and initiation sequences have been shown to affect transcription accuracy and efficiency (43, 44). Regardless, removal of the natural (+1), downstream (+34), and upstream (−12) initiation sites in construct IV resulted in complete loss of α-gene expression. These three sites are clustered within a 50-nt G+C-rich region that is bordered 5′ by an Sp1-like recognition sequence (the latter increases transcriptional accuracy in some genes; refs. 41 and 45). Although all three initiation sites are utilized in NB2a cells, transcription of the α gene occurs primarily, if not exclusively at position +1 in neural tissues (2, 12, 13, 46). Thus, these different sequences within the CK2 α-subunit gene promoter may function in concert to precisely and efficiently direct transcription initiation in a neuron type-specific and developmentally regulated manner.

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