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BC1 and BC200 scRNAs: Regulation of RNA polymerase III transcription in neurons and deregulation during tumorigenesis

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BC1 AND BC200 scRNAS: REGULATION OF RNA POLYMERASE III TRANSCRIPTION IN NEURONS AND DE-REGULATION DURING TUMORIGENESIS

BY

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ABSTRACT

BC1 AND BC200 scRNAS: REGULATION OF RNA POLYMERASE III TRANSCRIPTION IN NEURONS AND DE-REGULATION DURING TUMORIGENESIS

BY

WEI CHEN

Adviser: Associate Professor Jürgen Brosius

BC1 and BC200 are neuron specific cytoplasmic small RNAs conserved in rodents and primates, respectively. These two small RNAs come from different progenitors with different primary sequences, but they share several common characteristics. First, three similar domains are contained in the structures of BC1 and BC200 RNAs. Second, both RNAs are the first known RNA polymerase III transcripts with neuronal specific regulation. Third, the subcellular location of both RNAs is very similar; they have been identified in somatic and dendritic compartments of neurons. Fourth, both RNAs are part of a ribonucleoprotein complex (RNP). It has been hypothesized

that BC1 and BC200 RNAs might be involved in regulation of local postsynaptic protein biosynthesis in dendrites of nerve cells.

The main aspect of this work was to begin to elucidate the molecular mechanism which is responsible for the neuron-specific regulation of BC1 RNA. Transgenic mice carrying 1.4kb and 1kb (357bp including two octamer motifs were deleted at the 5′ end of the 1.4kb fragment) rat BC1 gene constructs have been studied. Both 1.4kb and 1kb transgenic mice expressed the transgene-encoded rat BC1 RNA in the nervous system but not in other tissues. Transgene BC1 RNA also has been detected in a subset of neurons where it is located in somatic and/or dendritic domains. Comparing these two constructs, we found that the levels of expression per transgene copy of deletion construct is about 5 times less than with the 1.4kb construct. In addition, the integration site-independence disappeared in the transgenic mice harboring the 1kb construct. We conclude that cis-acting regulatory elements, subject to neuron-specific control, are located within the 1kb construct of the rat BC1 RNA gene. Sequences in the 357bp deleted fragment might be candidates for elements responsible for transcriptional enhancement.

The second aspect of this work was to investigate the transcriptional regulation of both BC1 and BC200 RNAs during

tumorigenesis. These studies first demonstrated that both BC1 and BC200 RNAs are deregulated during certain neoplastic conditions. The induction of both small RNAs are specific for tumor cells and might play a role in the pathogenesis of those cancer tissues under some circumstances.

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CHAPTER 1

INTRODUCTION

Identification and Evolution of BC1 and BC200 RNAs

1. Identification of BC1 and BC200 RNAs.

BC1 RNA is a small cytoplasmic RNA and was discovered twelve years ago by Sutcliffe and co-workers (Sutcliffe et al., 1982) in rat brain. A striking characteristic of this small RNA is that it is prevalently expressed in the brain and exhibits a cytoplasmic distribution, hence the name: BC1 RNA. This small RNA was originally detected with a probe corresponding to a rodent short interspersed repetitive element (SINE), the so-called ID sequence. The 75 nucleotide ID sequence has 1-1.5x10^5 copies in the rat genome. These sequences were named "identifier sequences" because they seemed to mark hnRNA only in certain differentiated cells (Sutcliffe et al., 1984a, 1984b; Milner et al., 1984). At that time, it was believed that BC1 RNA represented a highly heterogeneous population, as a by-product of RNA polymerase III transcription of ID repetitive sequences within introns, such transcription being necessary for the RNA polymerase II transcribed brain-specific heterogeneous nuclear RNAs (hnRNAs) by RNA polymerase II. Since ID elements had initially been found being associated with neural genes, much attention was given to a role of ID repeats in cell type specific gene expression. Subsequently, ID elements were detected in various non-neural genes, and the

concept of the ID-dependent regulation of brain specific gene expression has been challenged (Owens et al., 1985; Sapienza and St.-Jacques, 1986; DeChiara and Brosius, 1987).

In order to determine if BC1 RNA represented the transcriptional by-product of various repetitive brain identifier (ID) elements or the independent transcript of a single or few genes, DeChiara and Brosius (1987) compared the sequences of cDNA clones. Since it was uncertain whether the A-rich region would represent the middle or the 3′ end of the BC1 RNA, priming the first-strand cDNA synthesis in the middle of the molecule was avoided by adding oligo (dC) to the 3′ end of polyadenylylated brain RNA. The majority of clones were identical in sequence and revealed three structural BC1 RNA domains: a 5′ domain was similar in sequence to the ID element followed by an internal A-rich region and a non-repetitive sequence domain was located at the 3′ end of each molecule. From blot-hybridization analysis, they found that an oligonucleotide probe of 30 residues complementary to the 3′ unique region of BC1 RNA only identified BC1 RNA but not hnRNA. Furthermore, only one band was detected in a Southern blot using the same probe. These results lead to the conclusion that BC1 RNA is transcribed from a single BC1 RNA gene.

Watson et al.(1987) reported that a BC1 RNA analogue, 200 nucleotide RNA in length and similar to the left monomer of Alu elements, was detected in monkey brain but not in non-

neural tissues using initially an ID sequence as a probe under low stringency. They named this BC1 analogue BC200 RNA. Although BC1 and BC200 RNAs have similar brain specific expression patterns, the sequences of the monkey and rat RNA products are very different.

Using the same strategy as for the isolation of the BC1 RNA (DeChiara and Brosius, 1987), a probe complementary to the sixty 3′-most nucleotides (the unique region including some A-rich region) of rat BC1 RNA was used to isolate human BC200 RNA cDNA clones at low stringency (Tiedge et al.,1993). Like rat BC1 RNA, human BC200 RNA can be subdivided into three structural domains. The 5′ region is similar to the left monomer of Alu repetitive elements following an A-rich region. The 3′ sequence is unique to BC200 RNA. Screenings of a genomic library using oligonucleotides specific to the 3′ unique region of BC200 RNA, three BC200 RNA genomic clones were isolated. One,BC200 α represented the active BC200 RNA gene. The other two were pseudogenes (BC200 β and γ) (Martignetti and Brosius, 1993a).

The comparison between BC1 and BC200 sequence indicated that the two genes had evolved via different phylogenetic routes. However, their neuron specific transcription and dendritic location may indicate similar functional roles of the RNAs in rodents and primates, respectively.

2. Evolutionary conservation of BC1 and BC200 RNA

BC1 RNA is likely derived from mammalian $tRNA^{Ala}$ (Daniels et al., 1985; Sakamoto et al., 1985b; Lawrence et al., 1985) via retroposition. There is 80% sequence similarity between the 5′ domain of BC1 RNA and mammalian $tRNA^{Ala}$. A secondary structure similar to mammalian $tRNA^{Ala}$ (Sakamoto et al., 1985a) can be proposed.

This small RNA is conserved only in rodents but not in other mammals during evolution (Martignetti and Brosius, 1993b). BC1 genes including the transcribed region and immediate flanking regions share high sequence similarities in all studied rodents (rat, mice, Syrian and Chinese hamster), even in the guinea pig which belongs to the Hystricognathi suborder of rodents sharing a common ancestor with the Sciurognathi suborder about 55 million years ago, the BC1 coding region is still 90% conserved while the more distal flanking regions are as expected highly divergent. The data strongly support the concept that there has been evolutionary pressure to maintain the BC1 gene for at least 55 million years. RNA blot analysis from all rodents examined indicates that the gene is also transcribed in a brain-specific manner for at least 55 million years.

The BC1 analogue, BC200 RNA in primates has arisen from an Alu repetitive element (left monomer), which in turn is derived from 7SL RNA (Ullu, et al., 1982, 1984). The BC200 gene is an example for a retroposon that was recruited as a new gene (Brosius, 1991; Brosius and Gould,1992). Since BC200 RNA is

present in Owl monkey (a new world monkey), which had a common ancestor with humans about 35 million years ago, the BC200 RNA coding sequence reveals 96% similarity between human and owl monkey. An active BC200 gene may have been generated between the mammalian radiation (60-100 million years ago) and 35 million years ago (Martin 1993; Martignetti and Brosius 1993a; B. Skryabin and J.Brosius unpublished observation).

Both BC1 and BC200 RNA genes are markers to trace the evolutionary relationship in rodents and primates, respectively.

Tissue Specific And Developmental Regulation Of BC1 And BC200 RNAs

1. Developmental regulation of BC1 RNA

BC1 RNA expression is under developmental regulation (Sutcliffe et al., 1984b). Rat BC1 RNA is only expressed in significant amounts after embryonic day 14. Then, BC1 RNA is detectable in the developing spinal cord. At day E15/16, BC1 RNA is detected in several brainstem areas. In the olfactory bulb, BC1 expression levels rise significantly at E 17/18 and reach peak levels shortly after birth. In the neocortex, appreciable BC1 RNA levels are detectable around birth, but a significant increase can be detected during the following weeks until constant levels are reached in the mature brain. A similar situation is seen in the hippocampus where BC1 expression levels begin to rise at the end of the first

postnatal week. In the cerebellum, BC1 expression in Purkinje cells (the only cell type in the cerebellum that expressed appreciable amounts of BC1 RNA) reaches maximum levels at the end of the first postnatal week, thereafter the expression drops to the relatively low levels in mature Purkinje cells (Liu V, Chen W, Brosius J and Tiedge H, unpublished observation). Interestingly, the developmental onset of BC1 expression in various areas of the rat nervous system coincides with the time of synaptogenesis in those areas.

2. Tissue specific regulation of BC1 and BC200 RNA

By using RNA blot techniques, several laboratories have reported that BC1 and BC200 are expressed in central and peripheral nervous systems of adult rodents and primates (Sutcliffe et al., 1982; 1984a; Tiedge et al., 1991,1993) but not in non-neural tissues, including liver, lung, kidney, spleen, skeletal and cardiac muscles. However, developing germ cells in male and female gonads were found to express BC1 RNA at appreciable levels.

The expression pattern of BC1 RNA in the adult rat nervous system was analyzed by in situ hybridization techniques (Tiedge et al., 1991). High levels of BC1 expression have been examined in gray matter areas, while little or no expression was in white matter areas throughout the brains. These strongly labeled brain regions included the neocortex, several thalamic and hypothalamic nuclei, the amygdaloid

complex, the superior colliculus, and several brainstem areas. In the spinal cord, only the central gray matter was labeled. At the cellular level, the density of silver grains was highest over the somata of large sensory neurons in the dorsal root ganglia. Adjacent cells showed much lower expression levels, and the lowest density was observed over fiber tract areas, and these anatomical structures contained axons of the sensory neurons and glial support cells.

The subcellular distribution of BC1 RNA in retina also revealed a high concentration of silver grains over the inner plexiform layer, whereas a weaker hybridization signal was evident in the ganglion cell layer as well as the inner most part of the inner nuclear layer. Over the outer nuclear layer and the outer plexiform layer little or no specific labeling was observed. The high levels of expression in the inner plexiform layer indicated an extrasomatic location of BC1 RNA. Because the inner plexiform layer contained a dense synaptic network with interactions among processes arising from bipolar cells, amacrine cells, and ganglion cells, the observed labeling patterns suggested that BC1 RNA may be located in at least some of these processes.

In cultured neurons , BC1 RNA expression was located in somata and almost all of the neuronal processes. This further supports that BC1 RNA has a somatodendritic location.

The distribution pattern of BC200 RNA in the human neural system is very similar to BC1 RNA, even at the subcellular

level (Tiedge et al., 1993).

The possible functions of BC1 and BC200 RNAs

There is evidence suggesting that protein synthesis may occur within dendrites in areas closely associated with the synaptic complex. First, polyribosomes are preferentially localized beneath synapses (Steward and Levy, 1992). Second, the number and localization of the polyribosomes are altered during the time of synapse formation in early development (Steward and Falk, 1986) or synapse replacement following lesions (Steward, 1983). Third, in general RNA is selectively transported into dendrites but not into axons (Garner et al., 1988; Bruckenstein et al., 1990; Kleiman et al., 1990). Examples of mRNAs in dendrites include the ones coding for MAP2, a high molecular weight form of the cytoskeletal protein MAP2 (Garner et al, 1988) which is a marker for the dendritic cytoskeleton (Matus et al, 1981;), and the one coding for the α-subunit of the calcium/calmodulin-dependent protein kinase II (Burgin et al., 1990), which is a major constituent of type I (asymmetric) postsynaptic membrane specializations (Kennedy. 1989). Fourth, active biosynthesis of proteins has recently been demonstrated in preparations of synaptosomes (Rao and Steward, 1991) as well as in preparations of dendrites (Torre and Steward, 1992). BC1 and BC200 RNA have approximately the same distribution as the mRNAs for MAPII and the α- subunit of CaM kinase II (Tiedge et al., 1991, 1993).

The similar anatomical and subcellular distribution of rat BC1 RNA and human BC200 RNA suggests equivalent functional roles of these RNAs in postsynaptic domains of various types of neurons in primates and rodents, respectively. These RNAs could be classified as functional analogues rather than as homologues due to their independent evolutionary histories. Most likely, the functional unit is a specific ribonucleoprotein particle (RNP). Based on recent experiments it is likely that BC1 RNA is complexed with proteins *in vivo* (Kobayashi et al., 1991, 1992, Cheng 1993, thesis). In addition, BC200 RNA contains a consensus sequence that is similar to the consensus sequence in 7SL RNA responsible for the binding of protein components of the signal recognition particle domain that functions in translation arrest (Walter, 1982). In the signal recognition particle, the protein heterodimer SRP9/14 is required for translational arrest. A sequence element similar to the SRP RNA consensus motif, responsible for binding of this heterodimer (Strub el al., 1991), is present in BC200 RNA. In the context of dendritic protein synthesis, such RNP may regulate translation-related processes. The arrest and re-initiation of postsynaptic translation for example would be dependent on synaptic activity. Differential protein biosynthesis may underly postsynaptic plasticity and thus memory and learning.

Transcriptional regulation of BC1 and BC200 RNAs

1.Promoters of RNA polymerase III

RNA polymerase III transcribes genes encoding highly conserved small RNAs required for protein synthesis (5S and tRNAs), for mRNA processing (U6 snRNA), for protein transport (7SL RNA), and for viral regulation of gene expression (adenovirus VA RNAs and Epstein Barr virus EBER RNAs) as well as some genes of unknown function (e.g. nuclear 7SK RNA).

Progress in the studies of the promoter organization of class III genes has been dramatic. First, the control regions of the *Xenopus laevis* 5S rRNA gene and later tRNA genes were found within the coding region (Bogenhagen et al., 1980, Sakonju et al., 1980, DeFranco et al., 1980, Galli et al., 1981). The opposite finding showed that the promoter domains essential for vertebrate U6 and 7SK genes were found entirely in the upstream sequence. Such an arrangement was typical of RNA polymerase II promoters (Murphy et al, 1987; Das et al., 1988, Folk 1988, Kunkel 1991, Nielsen et al., 1993). Finally, the combined elements from these two promoter classes were found in some class III genes such as the *Xenopus laevis* selenocysteine $tRNA^{(Ser)Sec}$ gene (Carbon and Krol, 1991).

Two major classes of internal control region containing genes have been identified; the first group included box A, and Box B. The tRNA genes and several viral small RNA genes share this type of promoter (Geiduschek and Tocchini-Valentini, 1988; Murphy et al., 1989, Willis 1993). The second group, which includes the 5S rRNA genes, had two components: Box A

which was identical to that of the first group, and Box C, which is unique to 5S rRNA genes.

A typical class III extragenic promoter with all promoter elements localized upstream of the transcribed sequence has been found in genes such as those encoding small nuclear vertebrate U6 RNA (snRNA) and human 7SK RNA. The whole coding region including a potential A box (there is no class III ICRs B box or C box within its coding region) can be removed with no effect on transcriptional efficiency. Three promoter elements have been identified (Folk 1988; Sollner-Webb, 1988; Kunkel, 1991). A TATA box is essential for transcription both *in vitro* and *in vivo*. It is similar in sequence and position to the TATA box found in polymerase II transcribed genes, and both motifs are functionally interchangeable (Kunkel, 1991; Lobo et al., 1991). A second element located upstream of the TATA box which displayed sequence similarity to the proximal sequence element (PSE), an essential promotor element of U1 and U2 snRNA polymerase II genes. PSE motifs from U6 and U2 genes were also interchangeable. Upstream from the PSE the U6 and 7SK genes contain sequences similar to the octamer elements or distal sequence element (around-250), which are enhancer like elements shared by class II snRNA genes, immunoglobulin light chain and histone H2B genes.

Both intra- and extragenic promoter elements were found in *Xenopus laevis* selenocystein $tRNA^{(Ser)Sec}$ gene. The upstream promoter was composed of a TATA motif, a PSE and a distal

element, SPH-like motif. An intragenic B box operated in synergy with those upstream elements and increased transcription efficiency. The natural A box of this gene was not functional (Carbon and Krol, 1991, Myslinski et al., 1992).

2. The transcriptional factors of RNA polymerase III

The fractionation of cellular extracts has revealed that in addition to RNA polymerase III at least two common protein factors are necessary for the transcription of all class III genes: TFIII C and TFIII B. TFIII A is required for the transcription of 5S RNA (Segall et al., 1980; Geiduschek and Tocchini-Valentini, 1988, Willis, 1993). During the initial transcription complex assembly, intragenic C box or B box is recognized by TFIII A or TFIII C, respectively. The association of those factors with the promoter forms a metastable complex which is stabilized by the addition of TFIII B (Geiduschek and Tocchini-Valentini, 1988).

TFIII B by itself has lower intrinsic affinity for DNA which appears to depend markedly on the template. The presence of a TATA box is likely to be important in demonstrating DNA-binding in the absence of assembly factors, TFIII C or TFIII A (Margottin et al., 1991). When polymerase III genes are without TATA boxes, TFIII B has not been reported to interact with DNA (Klekamp and Weil, 1986). However, this factor interacts with the TFIII C-tDNA complex or with the TFIIIA-TFIIIC-5SDNA complex and becomes precisely positioned just

upstream of the transcription start site (Kassavetis et al., 1990, 1991). Once TFIII B-DNA complex is formed, either with TATA-containing or TATA-less templates, then it becomes salt and heparin-resistant and is sufficient to correctly position polymerase III for multiple rounds of transcription in the absence of TFIII A and TFIII C (Kassavetis et al., 1990). TFIII B is the transcription initiation factor proper of RNA polymerase III while TFIII A and TFIII C are assembly factors. Recent studies show that a TATA-binding protein (TBP) plays a role in transcription by all three nuclear RNA polymerases (Comai et al., 1992, White et al., 1992, Cormack and Struhl, 1992, Sharp, 1992, Chiang et al., 1993) and TBP is a subunit of TFIII B (Willis 1993). A PSE-binding protein (PBP) is required for transcription of the mouse and human U6 genes in vitro (Waldschmidt et al., 1991, Simmen et al., 1991, Lobo etal., 1991).

3. Transcriptional regulation of BC1 and BC200 RNAs

BC1 RNA is the first example of a neural specific RNA polymerase III transcription product (Sutcliffe et al., 1984). From analysis of the genomic structure, it has been known that the BC1 gene contains intragenic promoters box A and box B (a variant) as well as extragenic promoter elements including TATA, PSE and two octamer binding sequences (Martignetti and Brosius, in preparation). Deletions of either box A or a variant box B alone, or in combination, eliminate

transcription *in vitro* using rat brain extracts. Deletion of extragenic promoters demonstrate that the two octamer sequences are not necessary *in vitro* but that the PSE and the TATA box are important for efficient BC1 RNA transcription in vitro. Furthermore, the BC1 B box, differing from the consensus B box of tRNAs by lacking an invariant A residue, represents a functionally related but distinct promoter element. The B box from BC1 RNA and tRNA are not functionally interconvertable. The wild-type BC1 B box is only maximally functional in the presence of 5′ flanking elements and was critically dependent on the spacing from it. A consensus B box is functional only after removal of the BC1 5′ flanking region. These results strongly suggest that juxtaposition of transcription factors, depending on the combination of intragenic and extragenic sequences, mediate either positive or negative interactions and are competing to form a transcriptional complex on the BC1 RNA gene template.

Like BC1 RNA, the BC200 gene contains intragenic promoters (A box and B box) as well as 5′ flanking elements (TATA box). Both are essential for BC200 RNA transcription in vitro using rat brain extract. In contrast to the BC1 gene, BC200 gene has an invariant A residue in its B box (a consensus B box).

The BC1 gene (with PSE, TATA, and box A+B′ necessary) belongs to a class III gene which differs from the 5S RNA genes (A box + C box necessary) or tRNA genes (A box + B box necessary), or from the 7SK and U6 RNA genes (OCT, PSE, TATA box necessary)

and also differs from the selenocysteine $tRNA^{(Ser)Sec}$ gene (SHP-like motif, PSE, TATA and box B necessary). This further indicates the complexities of transcription by RNA polymerase III. The unique feature of BC1 gene is that it must also contain additional cis acting element(s) essential for the nerve cell specific and developmental regulation. It represents an excellent model for the study of both polymerase III and neural-specific, developmental regulation. Studies towards identification of these elements are the main emphasis of this work (see chapter 3 and 4).

Transgenic Mice

Transgenic mice have been essential models for studying cell specific gene expression. Compared with cell transfection studies *in vitro*, this approach has the advantage of avoiding potential problems due to the transformed phenotype of cultured cells and allows direct comparison of expression in different tissues where the external gene is at the same location in the genome. Furthermore, the transgene is subject to developmental processes, such as DNA methylation and folding into higher order chromatin structures, which may strongly influence its expression (Felsenfeld, 1992).

In 1980, Gordon et al. first established a gene transfer system by pronuclear microinjection. The genes of interest were injected into the pronucleus of the fertilized mouse egg, stably and relatively randomly integrated into the mouse

genome. Therefore, the exogenous genetic material will appear in all cells of the newborn animal. They named this animal as "transgenic mouse". Based on the same principle, two other systems were established: retroviral infection (Jaenisch, 1976; Jahner et al., 1985) and embryonal stem (ES) cell transfer (Evans and Kaufman, 1981). In retroviral infection the size of the DNA fragment that can be packaged in recombinant retroviruses was limited and the expression of genes in such constructs was often poor. ES cells did not always populate the germ line, and the gene transfer protocol was more difficult than microinjection. Therefore microinjection became a widely used technique to identify DNA elements responsible for tissue-specific gene expression (Palmiter and Brinster 1986).

The frequency of gene transfer by microinjection is about 15-30%. Foreign genes may integrate as single copy or as head-to-tail concatemers (Costantini and Lacy, 1981), usually in a single site in the genome. Three aspects should be considered: cis-acting elements, trans-acting factors, and integration site. The expression pattern of transgenes is strongly influenced by those factors (Rosenfeld et al., 1988). Cis-acting elements are the most important in determining tissue specificity of expression. Cis-acting regulators referred to as promoters, enhancers, or promoter-enhancer elements which are usually located in 5′ regions of the genes. In the case of elastase-I, 205 bp of upstream DNA was

sufficient to direct expression of transgenes to the exocrine pancreas (Quaife et al., 1987). However, tissue-specific enhancers may also be located in the 3′ region of genes, (Behringer et al., 1987) and even located within coding regions (Behringer et al., 1987). Cis-acting elements interact with trans-acting factors forming DNA-protein complex resulting in gene activation. Conversely, repressor or silencer sequences may act to prevent expression in particular cells (Gorman et al., 1985) through modification of the positive trans-acting factors or binding to cis-acting regulatory elements. Thus, both positive and negative regulation may ultimately dictate the pattern of gene expression.

The chromosomal integration site commonly leads to very dramatic differences in both the level of transgene expression and sometimes the tissue specificity of expression (Brinster et al., 1981). These differences are attributed to cis-acting regulatory elements flanking the sites of integration. This effect may reflect the organization of chromosomes into functional domains which may be defined by special chromatin structures (Kellum and Schedl, 1991), by nuclear matrix attachments regions that may physically define chromosomal loops (Talbot. 1989; Stief et al., 1989. Webb et al., 1991), or by additional mechanisms that designate regions or single genes for repressive chromatin condensation. The most extensively studied cis-acting elements responsible for

position-independent is termed Locus Control Region (LCR) of the human β-globin gene cluster (Grosveld et al., 1987). LCR located about 20 kb upstream of the first gene in the β-globin cluster encompassing erythroid cell-specific DNaseI super-hypersensitive sites allows high levels and tissue-specific expression of the human β-globin gene independent of integration site on chromosomes (Talbot et al., 1989). A similar regulatory region is also found on the chicken lysozyme gene. The cis-acting regions flanking the chicken lysozyme-gene domain mediated the attachment of the chromatin to the nuclear scaffold. These 'A-elements' map to the 5' and 3' boundaries of the region of general DNase sensitivity in the active chromatin (Jantzen et al., 1986), which contain the lysozyme gene and its cis-regulatory elements. 'A-elements' can confer position-independent regulation to a whey acidic protein (WAP) transgene in mammary tissue of mice (McKnight et al., 1992).

Transgenic mice are powerful systems which allow studies of gene regulation in vivo. Correct tissue-specific expression patterns have been obtained for the human β-globin gene (Grosveld et al., 1987), the human CD2 gene (Greaves et al., 1990), the chicken lysozyme gene (Bonifer et al., 1990), the human MHC class I gene (Chamberlain et al., 1991) and many other genes including studies presented herein. (see chapter 3 and 5).

CHAPTER 2

METHODS

RNA Extraction From Tissues (Guanidinium/Cesium Chloride Centrifugation Method)

1. Five volumes of 5M Guanidine thiocyanate containing 25 mM Tris-HCl (pH 7.0), 10 mM DTT, 5 mM EDTA and 1% Sarkosyl was added to tissue and homogenized at room temperature for 60 seconds using a Polytron homogenizer. Insoluble debris was removed by centrifugation for 10 minutes at 2500 rpm.

2.Three ml of 5.7 M CsCl with 0.1 M EDTA (PH 7.0) was added into 12 ml capacity Beckman SW40 tubes and approximately 9 ml of the sample solution was layered on the top of the CsCl cushion.

3. The SW40 rotor was centrifuged for 20 hours at 15^0 (30,000 rpm). The supernatant was carefully removed and the inner wall was dried with Kimwipes. The pellet was dissolved in 0.4 ml of diethyl pyrocarbonate (DEPC) treated water with vortexing.

4. The RNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol, then was stored on dry ice for 15 minutes.

5. The RNA was collected by centrifugation at 15,000 rpm for 30 minutes at 4°C. The pellet was dissolved in DEPC-treated water, and the concentration was measured using the absorbance

at 260 nm.

Northern Blot Analysis

1.Labeling the Probes

The oligonucleotide probes HT005 and BC207 were designed for detection of BC1 and BC200 RNAs (Table 1.). HT005 is 60^{nt} complementary to 3′-most part of BC1 RNA and BC207 is 36^{nt} complementary to 3′-most part of BC200 RNA, respectively. 5′-end labeling with ^{32}P-ATP was catalyzed by T_4 polynucleotide kinase reaction (Maniatis et al., 1982).

Oligonucleotide probes used for Northern analysis of transgenic mice are shown in chapter 3. As above, they were labeled with ^{32}P. The Oligonucleotide probes for detection of 7SL RNA is 36^{nt} complementary to the middle non-Alu region of 7SL RNA. The same method was used for labeling.

The cDNA probe of rat cyclophilin (kindly provided by Dr.N.Levin) and rat β-actin (kindly provided by Wei Dong Jing) were labeled with ^{32}P by Nick translation reaction (Maniatis et al,. 1982) as a control for integrity and amount of RNA on the blots.

2. RNA Transfers

Total cellular RNA (10 μg) was size separaed on 1.8% agarose gels containing 2.2 M formaldehyde. The gel was directly transferred to a Gene Screen membranes (NEN) overnight with 10XSSC, then immobilized by UV-illumination (Church and Gilbert, 1984).

3. Hybridization

The RNA blot was prehybridized with prehybridization buffer in heat-sealable bags for 2 hours at 42°C for oligonucleotide probes, at 60°C for cDNA probes. This buffer contains 5X Denhardt's reagent, 0.5M Tris-HCl (pH 7.5), 1 M NaCl, 0.1% NaPPi, 1% SDS and 0.1 mg/ml yeast tRNA. The blot was hybridized by adding $1X10^6$ cpm/ml probe into the prehybridization bag overnight at 42°C for oligonucleotide probes, at 60°C for cDNA probes.

4. Washing the filters

The filter was washed in 0.1XSSC and 0.1% SDS at 65°C for β-actin, cyclophilin probes, at 55°C for HT005, 7SL, 7SK probes, in 0.5XSSC at 50°C for BC207 probe, and in 2XSSC at 45°C for RBC1 and MBC1 probes. The washing protocol was repeated for 3 times and lasted for 30 minutes each time.

5. Exposure of the film

The wet filter was wrapped in plastic wrap and exposed on Kodak X-Omat AR film in a cassette with screens at -70°C overnight.

6. Reprobing the filter

The filter was washed in 0.1XSSC and 0.1% SDS at 70°C for 2 hours to remove the probe. The filter was exposed to film overnight to examine whether the washing was complete. Then, the filter was used for reprobing.

Electrophoretic Mobility Shift

1.Preparation of the Probe for EMS

Sense and antisense strand of oligonucleotide probe of BC1w and BC1m were designed for EMS analysis (Table 1.). Double stranded probe was obtained by annealing both sense and antisense strand oligonucleotide in 0.8 M NaCl at 68°C for 15 minutes and then cooling to room temperature for several hours. Double stranded probes were then labeled with ^{32}P by T4 polynucleotide kinase reaction and purified on 6% polyacrylamide gel (19:1 acrylamide:bis) (Maniatis et al,. 1982).

2. EMS Analysis

Crude nuclear extracts from Hela cells (a gift from Dr. D. Anderson, Cal. Tech.) were performed in 16 μl final volume of reaction buffer containing 20 mM HEPES (pH 7.6), 0.1% NP-40, 10% glycerol, 1mM dithiothreitol, 2.5 mM $MgCl_2$, 250 mM KCl and 4 μg poly(dI-dC). Approximately 7 μg of the Hela extract was added to the reactive buffer and preincubated for 10 minutes at 4°C. Labeled DNA probe ($1X10^4$ to $2X10^4$ cpm per reaction) and competitors were then added following by 10 minutes incubation at room temperature. Electrophoresis was performed on a 3% polyacrylamide gel (37:1 acrylamide:bis) in 0.25 TBE for 3 hours at 200V at 4°C.

In Situ hybridization

1. Sample Preparation

Fresh tissues were quick frozen using liquid nitrogen and

embedded in Tissue-Tek OCT embedding medium and then sectioned in a Bright Microtome Cryostate at 10 μm thickness. Each sections was collected on gelatin and poly-L-lysine coated microscope slides.

2. Probe Preparation

60^{nt} and 36^{nt}double strand fragments which represent the 3'-ends of BC1 RNA and BC200 RNA (Table 1.), respectively, were subcloned into pBluescript KS (+) transcription vectors (Tiedge et al., 1991). ^{35}S-labeled RNA probes ("sense" and "antisense" strands) were transcribed from prelinearized templates using T3 or T7 RNA polymerase (BRL) (Melton et al.[Nucleic Acids Res 12: 7035,] 1984).

3. Hybridization

Thawed sections from -80°C were pre-fixed by fresh 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7-8) at room temperature for 15 minutes. Fixed sections were washed by 2XSSC (RNase-free) and illuminated by UV-light at 30W, 30cm distance for 15 minutes. Hybridization was performed at 50°C overnight with hybridization buffer. This buffer contained 50% deionized formamide, 0.6 M NaCl, 10 mM Tris HCl pH 7.5, 0.02% each Ficoll, PVP, BSA, 1 mM EDTA, 0.5 mg/ml ssDNA, 0.6 mg/ml total yeast RNA, 10% dextran sulfate. Each section was carefully covered by the buffer. The probe concentration was 3-5X10^{6} cpm/ml.

4. Washes

Low stringency wash was performed in 2XSSC at 40^{0}C. Then

unhybridized RNA was digested with 30 μg/ml RNase A at 37°C for 1 hour. The final high stringency wash was completed in 0.1XSSC at 55°C for the BC1 probe and in 0.1XSSC at 37°C for the BC200 probe, respectively.

5. Handling Emulsion

After washing, dried slides were exposed on film which needed one day for the BC1 probe and 10 days for the BC200 probe. Then, slides were dipped in NTB_2 emulsion (Kodak) at 42°C for 2 seconds. Dipped slides were dried in air for 2 hours and exposed at 4°C for 5 days (BC1 probe) or 3 weeks (BC200 probe).

6. Developing

Slides were developed for 4 minutes in D19 developer (15°C), then rinsed with H_2O for 30 seconds. A Kodak fixer was used to fix these slides for 5 minutes. These slides was washed three times in H_2O, each wash for 5 minutes. Finally, these slides were stained with cresyl violet and coverslips were mounted.

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDE PROBES USED IN THE STUDIES

OLIGO.	PROBE	OLIGO. SEQUENCES
BC207	36mer	5'CTTGTTGCTTTGAGCGAAGTTACGCTTATTTGGTAC3'
HT005	60mer	5'AAAGGTTGTGTGTGCCAGTTACCTTGTTTTTTTTTGGTCTTTTTGTTATT TTGTCTTTTT3'
7SL	36mer	5'CGAGGTCACCATATTGATGCCGAACTTAGTGGGTAC3'
RBC1	17mer	5'TTTTTTCGGAGCTGAGG3'
MBC1	17mer	5'TTTTTCCAGAGCTGAGG3'
BC1w	30mer	5'TTTTCGGAGCTGAGGACCGAACCCAGGGCC3'
BC1m	30mer	5'AGAAGCTTGCTGAGGACCGAACCCAGGGCC3'
S36	36mer	5'CAAAGCCATTTCAGCACCACGGAGAGTGCCTCTGC3'
CRE	36mer	5'TCGACGGATTGAATTCAGGCCGTCAGATTC3'

CHAPTER 3

NEURAL SPECIFIC EXPRESSION OF RAT BC1 RNA GENE IN TRANSGENIC MICE

ABSTRACT

Rodent BC1 RNA is a 152-nucleotide-long, nontranslatable RNA that is almost exclusively expressed in nerve cells and located in the dendrites and somata of a subset of neurons in the central and peripheral nervous system. It is the first known RNA polymerase III transcript with neuronal specificity and developmental regulation. To gain insight into potential cis- and trans-acting factors defining neuronal identity, we generated transgenic mice carrying a 1.4 kb DNA fragment containing the rat BC1 gene. The fragment contains the transcribed region plus 429 bp of 5′-flanking and 870 bp of 3′-flanking sequences with RNA polymerase II and III consensus promoter sequences. Three transgenic lines were analyzed. All expressed the transgene-encoded BC1 RNA in the nervous system but not in other tissues. Transgene BC1 RNA also has been detected in a subset of neurons where it is located in somatic and/or dendritic domains. The cell-type specific expression of the rat BC1 RNA in transgenic mouse brain was independent of the sites of integration. These results suggest that cis- acting regulatory elements, subject to neuron-specific control, are located within the 1.4 kb fragment of the BC1 RNA gene.

INTRODUCTION

Brain specific cytoplasmic small RNA (BC1) is the first example of a neuron specific RNA polymerase III transcription product under developmental regulation in rodents (Sutcliffe et al., 1982). The BC1 RNA gene is derived from $tRNA^{Ala}$ and has existed for at least 55 million years (Daniels and Deininger, 1985; Sakamoto and Okada, 1985; Lawrence et al., 1985; Martignetti et al., 1993). There are three structural domains in BC1 RNA: a 5′ domain similar in sequence to the ID element (for which BC1 RNA is a source gene [Kim et al., 1994]) followed by an internal A-rich region and a non-repetitive sequence domain located at the 3′ end of each molecule (DeChiara and Brosius, 1987). The subcellular location of this small RNA has been determined in both somatic and dendritic compartments of a subset of neurons (Tiedge et al., 1991). It has been suggested that BC1 RNA is likely to be complexed with proteins to form a ribonucleoprotein particle (Kobayashi et al., 1991, 1992; Cheng 1994 thesis). BC1 RNA expression coincides with synaptogenesis in the developing nervous system and primary neuronal cultures (H.Tiedge, personal communication). Therefore it has been hypothesized that BC1 RNA might play a functional role in local postsynaptic protein biosynthesis in neurons (Tiedge et al., 1991).

Our recent studies have shown that the BC1 RNA gene is conserved in rodent species both with respect to primary sequence and neural-specific expression pattern (Martignetti,

thesis 1992). The conserved sequences include both RNA polymerase III intragenic promoters; namely, A and B boxes as well as RNA polymerase II upstream promoter elements consisting of two octamer binding sequences, a proximal sequence element (PSE), and a TATA box (Martignetti and Brosius, in prepararion). We have also demonstrated that both pol II and pol III promoter elements are essential for BC1 RNA transcription in a whole cell rat brain extract system. Deletion or alteration of the internal A or B box sequence or entire flanking upstream region abolishes transcription while alteration of the TATA and PSE sequences greatly reduce transcription efficiency (Martignetti and Brosius in preparation).

Little is known about the precise role of the various cis-acting DNA elements in the neuronal specific regulation of BC1 RNA. In this report, we show that a 1.4 kb fragment containing the rat BC1 gene was sufficient for neural-specific transcription when introduced into the mouse genome. This fragment contains transcribed region plus 429 bp of 5′ flanking sequence and 870 bp of 3′ flanking sequence with both polymerase II and III promoters. The expression of the transgene is selective for the nervous system and is independent of the integration site. The results of these experiments indicate that neuron-specific cis-acting sequences are present within the 1.4 kb BC1 gene fragment.

METHODS

Preparation and analysis of genomic DNA

Genomic DNA (10μg), extracted from the tails of 14 candidate transgenic mice, was digested with EcoR I and size fractionated on an 1.2% agarose gel and transferred to nylon membrane for Southern blot analysis. The membranes were then hybridized with a ^{32}P-labelled probe corresponding to the upstream region (position -423 to -5) of the rat BC1 RNA gene. Hybridization was performed at 60°C in 5X SSPE, 0.5% sodium dodecyl sulfate (SDS) for 16-18 hours. The membranes were washed three times at 65°C in 0.1X SSC, 0.1% SDS for 30 min. Transgene copy number was estimated by dot blot hybridization (Palmiter et al., 1983) with the ^{32}P-labelled oligonucleotide probe corresponding to the 3'-unique region of BC1 RNA followed by quantitation using phosphorimage scanning from several experiments.

Preparation and analysis of RNA

Rat BC1 DNA positive offspring from founders #54, #59 and #60 were sacrificed and fresh tissues were removed and immediately frozen in liquid nitrogen. Total RNA was isolated by homogenization in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion. RNA (10μg) was fractionated on 1.8% agarose-formaldehyde gels for Northern blot analysis (Maniatis et al., 1982). The oligonucleotide

probes (fig.3) were labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Hybridization was performed at 37°C in 1M NaCl, 0.5M Tris-HCl (pH 7.5), 5X Denhardt's reagent, 1% SDS and 0.1mg/ml yeast tRNA . The membranes were then washed three times at 45°C in 2X SSC and 0.1% SDS for 30 min. The levels of transgene expression were then quantitated by phosphorimage scanning from several experiments.

In Situ hybridization

Fresh brain tissue from positive offspring were quick frozen using liquid nitrogen, embedded in Tissue-Tek OCT embedding medium, and then sectioned in a Bright Microtome Cryostat at 10 μm thickness. Sections were collected on gelatin and poly-L-lysine coated microscope slides.

A transcription vector (pMK1) containing a sequence that represent the 60 nucleotides of 3' most region of BC1 RNA was subcloned into pBluescript KS(+) transcription vectors (Tiedge et al., 1991). ^{35}S- labelled RNA probes ("sense" and "antisense" strands) were transcribed from prelinearized templates using T3 or T7 RNA polymerase (BRL protocol).

In situ hybridization experiments were performed as previously described (Tiedge, 1991). This protocol uses UV- light as cross linking agent to improve signal-to-background ratios.

RESULTS

Production of transgenic mice carrying a rat BC1 gene.

A 1.4 kb DNA fragment containing 429 bp of 5′ flanking sequence and 870 bp of 3′ flanking sequence of the rat BC1 gene was isolated from plasmid BC1:KS by SacI and BamHI digestion (Fig.1). Purified DNA was prepared and given to the Mount Sinai Transgenic Facility for microinjection. DNA extracted from the tails of 14 candidate transgenic mice was analyzed for the presence of the transgene by Southern hybridization with a ^{32}P labeled probe corresponding to the upstream region (from position -423 to -5 of the rat BC1 gene). Three mice carrying the BC1 transgene were identified (Fig.2). Copy number was estimated by quantitative dot blot analysis (see Materials and Methods). The three founder mice (#54, #59 and #60) carried between 14-20 copies each of the BC1 transgene (Data not shown). All three founder mice were crossed with wild-type C57BL/6 mice.

The rat BC1 transgene is expressed in brain and testes.

To determine whether the 1.4 kb fragment of the rat BC1 gene contained sufficient regulatory information to direct the neuron-specific expression of BC1 RNA, total RNA from different tissues of offspring of each founder was analyzed by Northern blot hybridization. RNA from rat brain and wild-type C57BL/6 mouse brain was used as positive and negative controls, respectively. Since rat and mouse BC1 RNAs share more than 98% sequence similarity, there is only a 2 bp

difference between the entire rat and mouse BC1 RNA sequences. In order to distinguish between endogenous mouse and transgene-encoded rat BC1 RNAs, two specific oligonucleotide probes were designed (Fig.3): the rat BC1 probe was complementary to transgene-encoded BC1 RNA and has a 2 bp mismatch with endogenous mouse BC1 RNA; the mouse BC1 probe was complementary to endogenous BC1 RNA but has a 2 bp mismatch with the rat BC1 transgene RNA. In the three transgenic lines (#54, #59 and #60) rat BC1 RNA was detected in brain by the rat BC1 probe but not in liver and lung ; however, as expected low levels of BC1 RNA was detected in testes (Fig.4). Other non-neuronal tissues including kidney, heart, spleen and lymphnode have been examined; BC1 RNA was not present in those tissues which was shown for line #59 in figure 5. A cDNA probe for the ubiquitously expressed cyclophilin mRNA was included as a control for integrity and amount of RNA on the blots (Fig.4 bottom panel). The levels of BC1 transgene RNA were about 125-350% of endogenous BC1 RNA. The highest level was found in line #60 which contains the highest transgene copy number. The lowest transgene transcript level was observed in mice from line #59 which contained the fewest copies of the transgene. The data indicated that rat BC1 gene is efficiently expressed in transgenic mouse brain with a distribution pattern which is similar or identical to endogenous BC1 RNA and independently of the sites of integration.

Transgene expression is neuron-specific

To determine the transgene RNA product distribution at the cellular level, in situ hybridization was performed. An ^{35}S-labeled antisense RNA probe was generated complementary to a portion of the 3′ unique domain of BC1 RNA which detects both endogenous and BC1 transgene RNA. A sense strand probe, corresponding to this portion of BC1 RNA, was used as a negative control. In general, a similar expression pattern is seen in nervous system between transgenic and non-transgenic mice. However, a higher level of BC1 RNA was detected in transgenic mouse brain than in non-transgenic mouse brain (data not shown). These data indicate that the expression patterns of rat RNA and endogenous mouse BC1 RNA appear to overlap extensively if not completely: BC1 RNA labeling was restricted to gray matter areas including the neocortex, several thalamic and hypothalamic nuclei, amygdaloid complex, superior colliculus and several brainstem areas. No labeling has been shown in white matter areas which contain axonal bundles and glial cells. The labeling patterns described in these studies are the same as for endogenous rat BC1 RNA (Tiedge et al., 1991).

The BC1 RNA labeling pattern in transgenic mouse lines #54, #59 and #60 was closely examined in the olfactory bulb and hippocampal formation. The result for line #60 is shown in Figure 6 and 7. The region of the olfactory bulb consists of

laminar structure. Neuronal cell bodies are concentrated in one layer and the dendrites are located in another layer (Fig.6a and 6b). Labeling was observed crossing the whole external plexiform layer and mitral body layer, whereas less BC1 signal appeared in the granular cell layer (Fig.6d). The external plexiform layer contains the majority of dendrites from granule cells, mitral cells and tufted cells, and these cells form a dense synaptic network (Fig.6a and 6b). "Sense" control was shown on figure 6c. In the CA3 field of the hippocampus (Fig.7), BC1 labeling was most intense in the stratum pyramidale , the layer that contains somata and the proximal dendrites of pyramidal cells. Strong labeling was also observed over the stratum oriens and the stratum radiatum. These layers contain the basal and the distal apical dendrites of the pyramidal cells, respectively. These anatomical data with the observed labeling pattern indicate that, similar to endogenous mouse BC1 RNA, rat BC1 transgene RNA is located both in somata and dendrites of neurons. This suggests that all necessary elements for cell type specific expression are located within the 1.4 kb rat BC1 gene.

DISCUSSION

Neural-specific expression probably occurs, by analogy with other tissues, primarily at the transcriptional level, through interactions between cis-acting DNA elements and transcription

factors located in the nervous system (Johnson and McKnight 1989; Struhl 1991; Mandel and McKinnon 1993;). However, identification of the DNA elements and transcription factors responsible for expression in the nervous system has been less forthcoming. As a step toward understanding the mechanisms regulating neuron-specific gene expression, we chose to study the regulatory sequences of the neuron-specific BC1 gene. Rodent BC1 RNA represents the first example of a neural-specific RNA polymerase III transcription product, and therefore provides a model for the study of both polymerase III and neuron-specific transcriptional regulation.

It has previously been reported that BC1 RNA expression patterns are deregulated in cultured rodent cell lines (McKinnon et al.,1986). Our recent study also shows that unlike whole tissue extracts, the extracts prepared from cultured cells are less stringent in their requirements for the upstream BC1 promoter elements and are non-dependent on the interactions between intragenic and extragenic promoters (Martignetti et al., in preparation). Cultured cell lines are generally poorly differentiated and only a few reports have suggested that tissue specificity might be reproduced under in vitro conditions (Tsuda and Suzuki, 1981; Scholer and Grass, 1985). In order to avoid this disadvantage of in vitro systems, transgenic mice carrying the rat BC1 gene have been produced and analyzed. The primary advantage of this technique

is that expression of the transgene can be tested in the complete range of fully differentiated cell types found in the mouse as well as during development.

Introduction of the 1.4 kb rat BC1 gene into the mouse germline has resulted in efficient levels of expression in three independent transgenic mouse lines and, with the exception to lower levels in the testes that is also observed in nontransgenic mouse, it was restricted to the nervous system. It was not found in liver, lung, spleen, heart, kidney or other non-neuronal tissues. The expression pattern of the transgene and endogenous BC1 gene was shown to be parallel even with respect to their subcellular localization: dendrites and somata of neurons. These results suggest that cis-acting regulatory elements sufficient for targeting of neuron-specific expression are located within the 1.4 kb of the rat BC1 gene. In addition, a similar pattern of expression in all three transgenic lines indicates that transgene expression appears to be independent of chromosomenal integration sites.

It has been reported that a buffer or boundary sequence on both sides of a transcriptional unit might be required to isolate a modestly active gene from cis-acting effects of the sites of integration and from transcriptional interference between duplicated copies of the gene arranged in head-to-tail

fashion (Proudfoot, 1986; Ryan et al., 1989; Bonifer et al., 1990; McKnight et al., 1992; Thorey et al., 1993). In these studies, we observed the average levels of expression per copy of the rat BC1 gene were about 27% of endogenous mouse BC1 RNA levels. It is possible that some copies of the gene are particularly active while others are silent. In addition, the possibility of increasing degradation of overexpressed BC1 RNA in transgenic mouse brain also could not be ruled out. Unfortunately, the absence of single-copy BC1 transgenic animals prevents a conclusion concerning whether all transgene copies are equally active. The results do not rule out that other flanking sequences might be necessary for expression of each transgene copy at the same level as the endogenous mouse BC1 gene.

A disagreement could arise from the data of in situ hybridization concerning the subcellular location of transgene BC1 RNA, since the probe is able to detect both endogenous and transgene BC1 RNA. We primarily aimed to identify whether the transgene was expressed in the same cell types as the endogenous mouse BC1 RNA gene. It is difficult to distinguish a 2 bp mismatch between both RNAs using in situ hybridization techniques. The conclusion of overlapping expression of rat and mouse BC1 RNA is based on following evidence: First, BC1 RNA labeling in transgenic mouse brain was proportionally higher than in non-transgenic brain. No ectopic expression was observed in transgenic mouse brain. Second, using rat and

mouse specific oligonucleotide probes, we are able to detect both rat transgene BC1 and endogenous mouse BC1 RNA in transgenic mouse brain by Northern blot analysis. As functional homologs, it is not surprising that the expression patterns of both RNAs are, to the extent that they have been examined, strikingly similar. For example, in the hippocampus, both RNAs are expressed in pyramidal cells in Ammon's horn and in the hilar region. Significant BC1 labeling in dendrite-rich areas of olfactory bulb suggests that both RNAs are located in somatodendritic domains. Our recent studies have shown that primate BC200 RNA is prevalently expressed in the nervous system (Tiedge et al., 1993). BC200 RNA and BC1 RNA seem to be expressed by equivalent subsets of neurons in the primate and rodent nervous system, however they are not classified as homologues but as functional analogs.

Transcriptional regulation of the rat BC1 gene has been extensively studied in our group using whole cell extracts (Martignetti et al., in preparation). We demonstrated that BC1 gene belongs to a class III gene and both intragenic (A and B Boxes) and potential 5' flanking elements (TATA, PSE) of the gene are important for its transcription in vitro. Data from these studies further confirmed that the 1.4 kb rat BC1 gene is not only efficiently transcribed in vitro but also in vivo with appropriate tissue specificity. It is not clear which sequences within the 1.4 kb construct are responsible

for BC1 RNA gene neural-specific expression. To answer this question, further deletion and mutation of this construct will be employed to localized those sequences. Taken together, the efficient and appropriate tissue-specific expression of the 1.4 kb rat BC1 gene fragment in transgenic mice provides the potential opportunity for identifying sequences which participate in neuron- specific regulation with particular emphasis on RNA polymerase III transcribed genes.

Figure 1: Structure of rat BC1 RNA gene construct.

Schematic diagram of the rat BC1 RNA gene, 1.4 kb construct, used to generate the transgenic mice. The upstream and coding region promoter elements are as noted above.

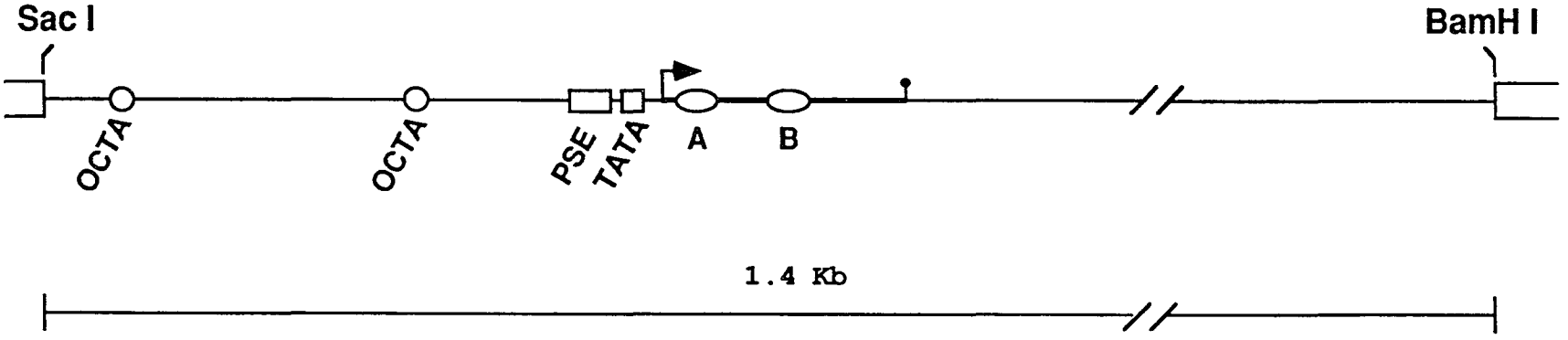
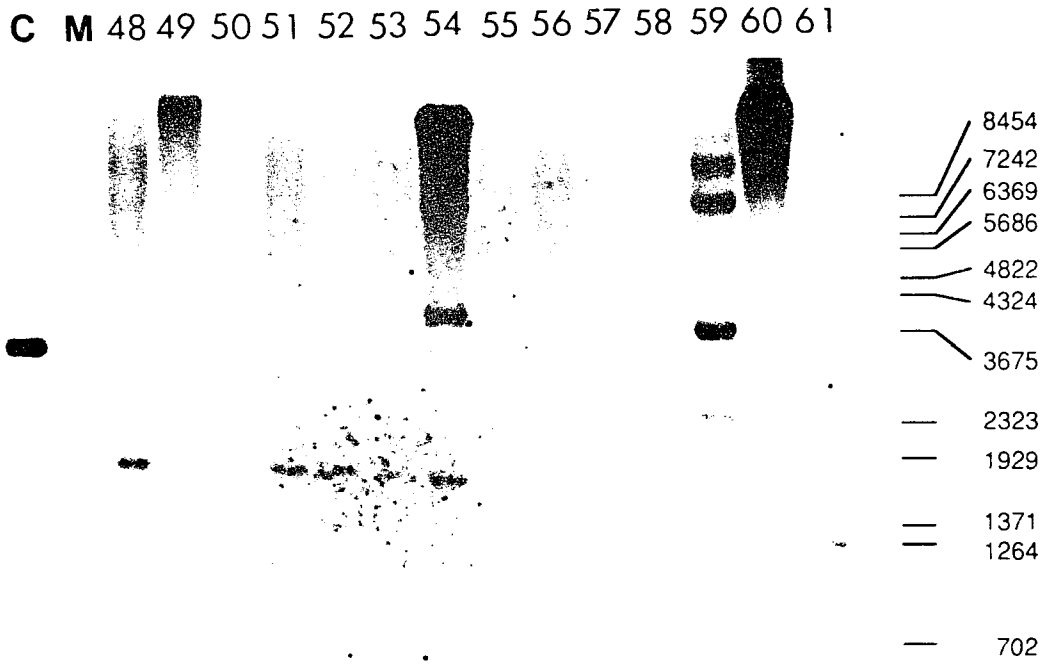

Sac I
BamH I
OCTA
OCTA
PSE
TATA
A
B
1.4 Kb

Figure 2: Southern Blot Analysis of the DNA from 14 Candidate mice.

Approximately 10 μg of each DNA sample, extracted from 14 candidate transgenic mice tails, was digested with EcoRI, separated on a 1.2% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labelled probe (upstream region from position -423 to -5 of the rat BC1 RNA gene). The endogenous mouse BC1 RNA gene, internal control, corresponds to 1.7 kb fragment seen in all samples. Three BC1 positive transgenic mice, #54, #59 and #60, are characterized by the presence of additional bands. A plasmid (C) containing the upstream region of rat BC1 RNA is shown on the left while size standards (M) are indicated to the right. This Southern analysis was performed by J. Martignetti.


C M 48 49 50 51 52 53 54 55 56 57 58 59 60 61
8454
7242
6369
5686
4822
4324
3675
2323
1929
1371
1264
702

Rat Probe	**3'GGAGTCGAGGCTTTTTT5'**
	*** ***
Mouse BC1	**5'CCUCAGCUCUGGAAAAA3'**
Mouse Probe	**3'GGAGTCGAGACCTTTTT5'**
	*** ***
Rat BC1	**5'CCUCAGCUCCGAAAAAA3'**

Figure 3: Probes for RNA blot analysis are designed to distinguish between the endogenous mouse BC1 RNA (MBC1) and transgene rat BC1 RNA (RBC1). The respective rodent probes are specific for either rat or mouse BC1 RNA by possessing a 2 bp mismatch with the other RNA. Asterisks (*) indicate these two mismatches.

Figure 4: Northern Blot Analysis of rat BC1 RNA Expression in Transgenic Mice.

Total RNA (10 μg per lane) extracted from transgenic mice (#54, #59 and #60) brain (B), liver (L), lung (Lu), testis (T), and from nontransgenic mouse brain (MB) and rat brain (RB) was separated on a 1.8% agarose-formaldehyde gel. It was then blotted onto a nylon membrane, and hybridized with ^{32}P-labelled rat BC1 (top panel), mouse BC1 (middle panel) and cyclophilin (bottom) probes, respectively.

Using mouse BC1 probe, a positive signal was detected in non-transgenic mouse brain (MB) and in all three transgenic mouse brains (B) which represent endogenous mouse BC1 RNA. A weak signal was also detected in testes of #54, #59 and #60 (T). No signal was detected in rat brain and other tissues. Using rat BC1 probe, a positive signal was detected in rat brain (RB) and in #54, #59 and #60 transgenic mouse brains (B), respectively. As endogenous mouse BC1 RNA, a weak signal was also present in all transgenic mice testes (T). Rat BC1 RNA was not present in nontransgenic mouse brain (MB) and other tissues. Cyclophilin probe hybridized to all samples with similar intensities.

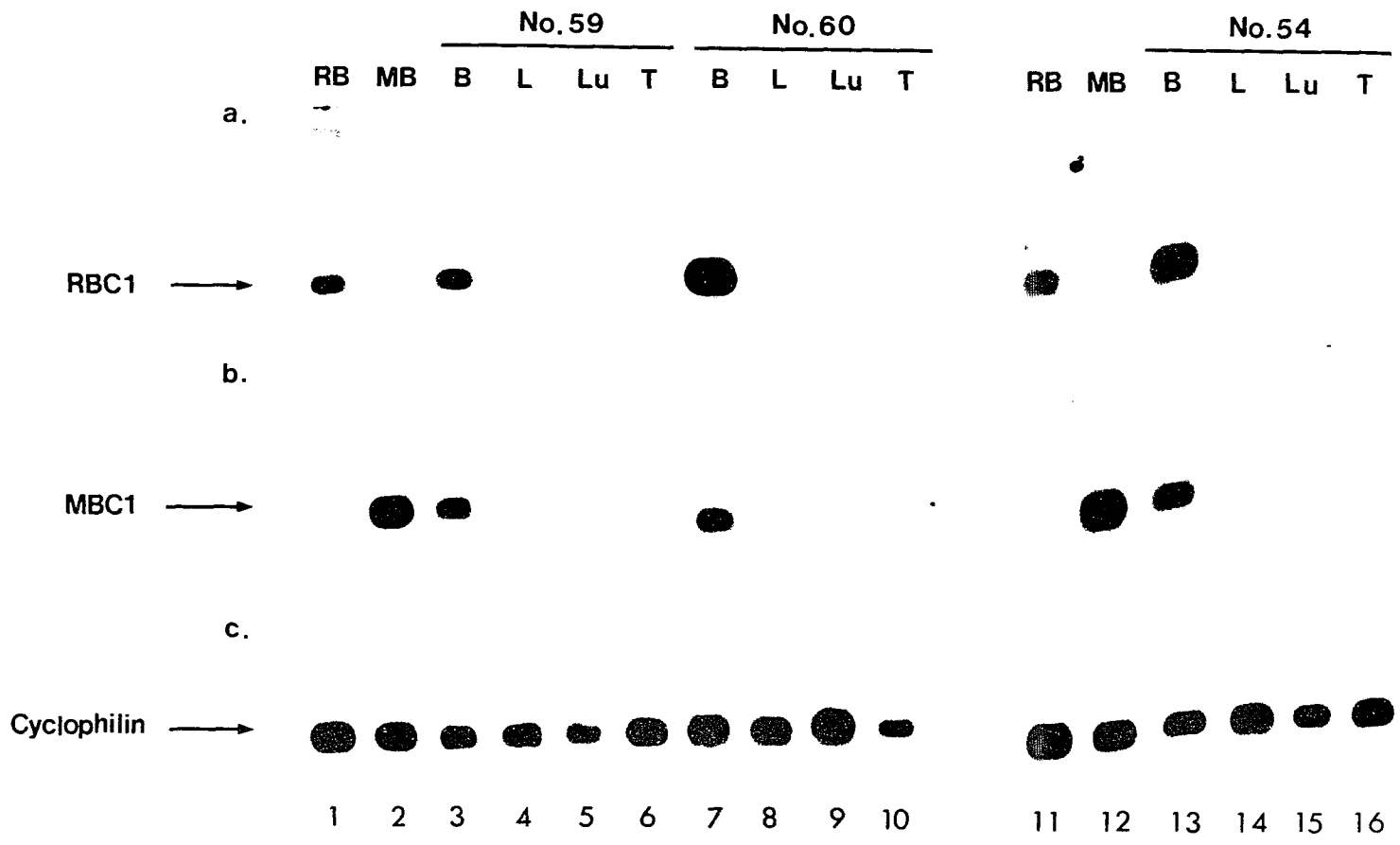

No. 59
No. 60
No. 54
RB
MB
B
L
Lu
T
a.
RBC1
b.
MBC1
c.
Cyclophilin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 5: The expression pattern of transgene rat BC1 RNA in transgenic mouse line #59.

Total RNA (10 μg per lane) was extracted from line #59 brain (B), kidney (K), heart (H), liver (L), lymphnode (Ly), spleen (S), nontransgenic mouse brain (MB) and rat brain (RB) and was size separated on a 1.8% agarose-formaldehyde gel. It was then blotted onto a nylon membrane, and hybridized with ^{32}P-labelled rat BC1, RBC1 (top panel), mouse BC1, MBC1 (middle panel) and cyclophilin (bottom panel) probes, respectively. Using mouse BC1 probe, a positive signal was detected in non-transgenic mouse brain (MB) and #59 transgenic mouse brain (B) but not in other tissues. When using rat BC1 probe, transgene rat BC1 RNA was detected in rat brain (RB) and #59 transgenic mouse brain (B) but not in other tissues. A weak signal was also detected in non-transgenic mouse brain due to cross hybridization of the rat probe (G:U and G:T juxtapositions as mismatchs). Cyclophilin probe hybridized to all samples with similar intensities.

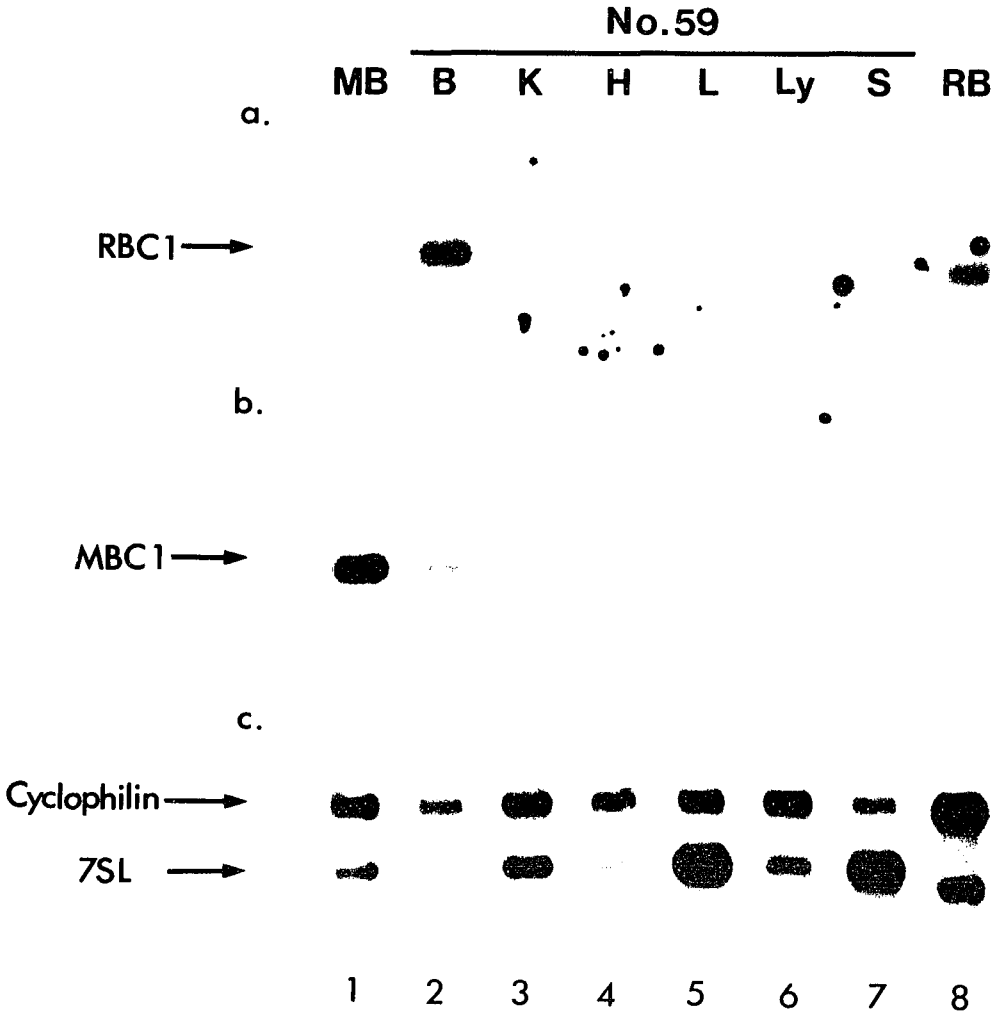
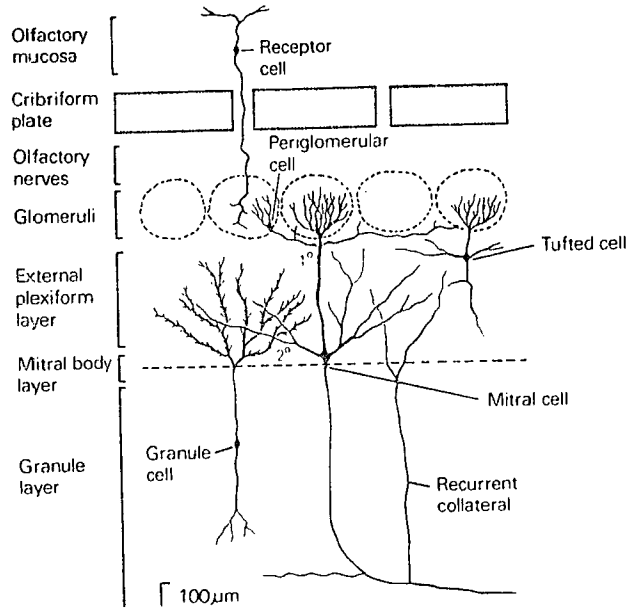
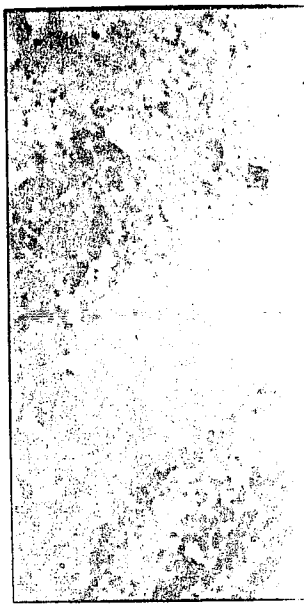

No.59
MB
B
K
H
L
Ly
S
RB
a.
RBC1
b.
MBC1
c.
Cyclophilin
7SL
1
2
3
4
5
6
7
8

Figure 6: Subcellular Distribution of BC1 RNA in Transgenic Mouse Line #60 Olfactory Bulb.

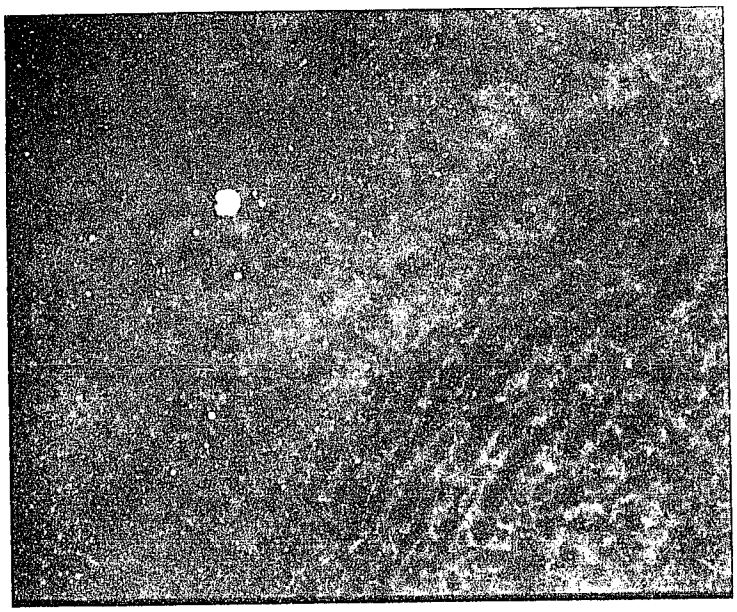
Panel a and b: The laminar structure of olfactory bulb is shown: glomeruli (GL), external plexiform layer (EPL), mitral body layer (MBL), granule layer (GrL). Panel c: ^{35}S-labelled "sense strand" RNA probe, corresponding to the 3′ most sequence of BC1 RNA, was used to detect nonspecific labeling. Panel d: ^{35}S-labelled "antisense" RNA probe was complementary to the 3′ unique region of BC1 RNA. White silver grains indicate BC1 RNA labeling intensities. Strong labeling is observed throughout the EPL which contains majority dendrites, and in MBL which contains mitral cells. Less signal appear in GrL and in GL. The former contains axon and granule cells. Bright-field (b) and dark-field (c and d) micrographs were taken with a Nikon microscope. Scale bar, 30 μm. Panel a is taken from the text book "Principles of Neural Science".

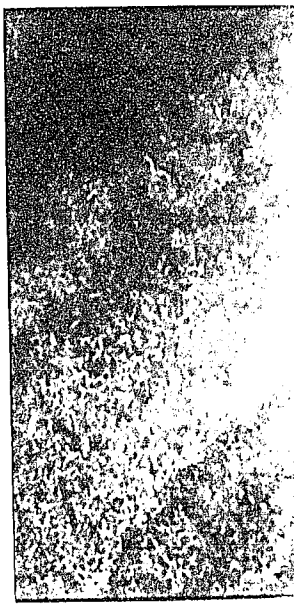
a.

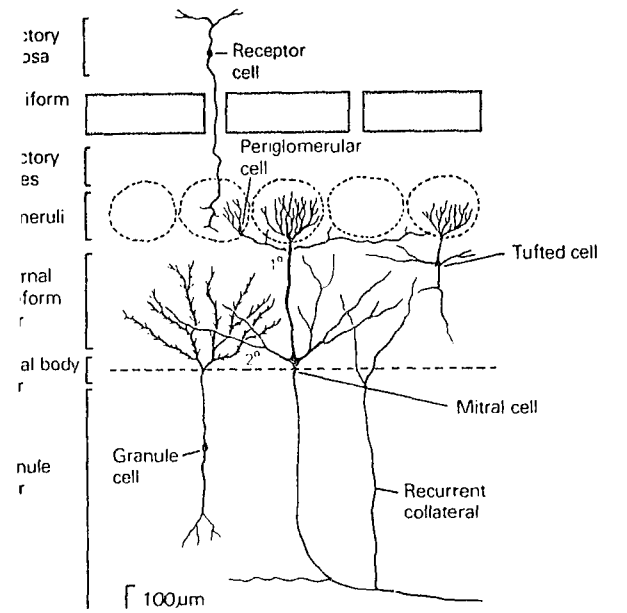

Olfactory mucosa
Receptor cell
Cribriform plate
Periglomerular cell
Olfactory nerves
Glomeruli
Tufted cell
1°
External plexiform layer
2°
Mitral body layer
Mitral cell
Granule cell
Granule layer
Recurrent collateral
100 μm

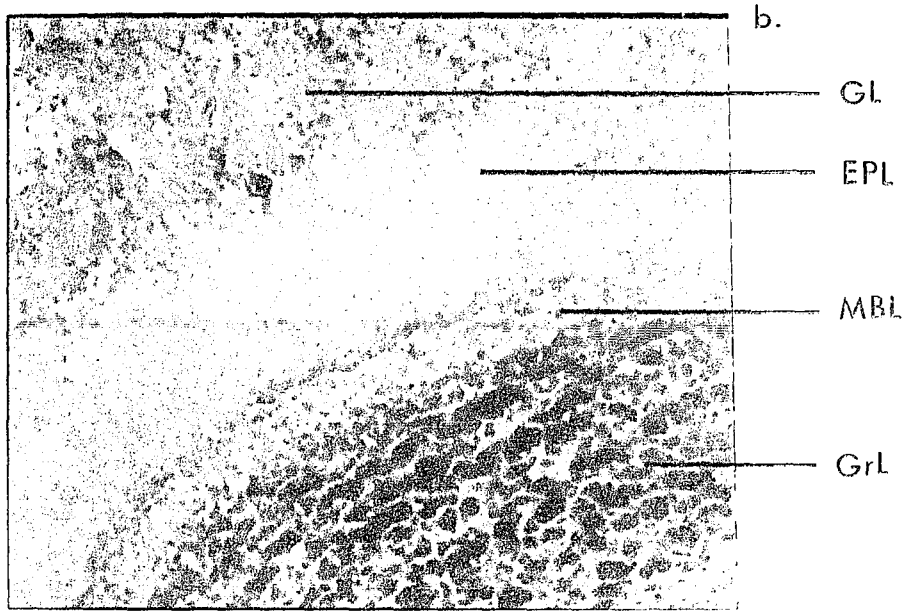


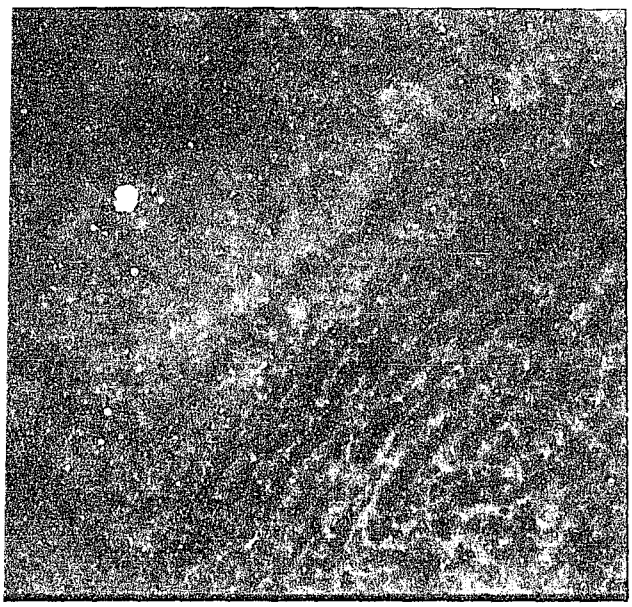
c.






Receptor cell
Periglomerular cell
Tufted cell
Mitral cell
Granule cell
Recurrent collateral
100μm


b.
GL
EPL
MBL
GrL



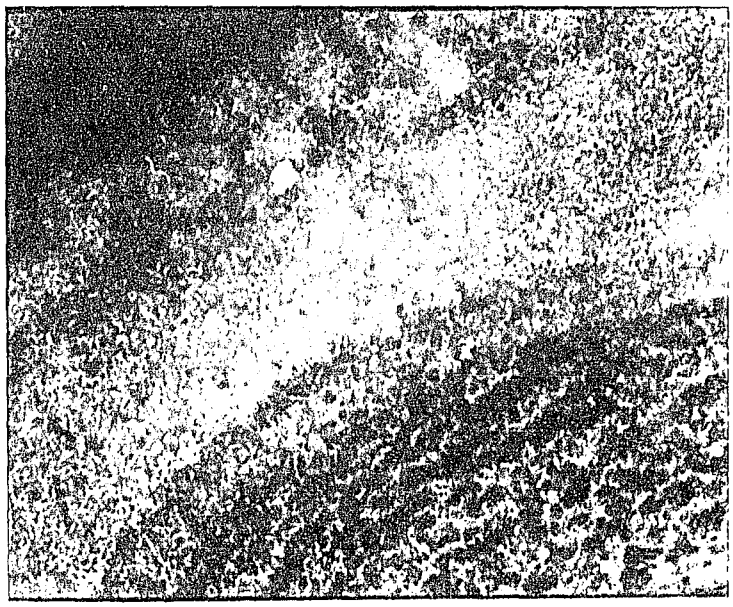
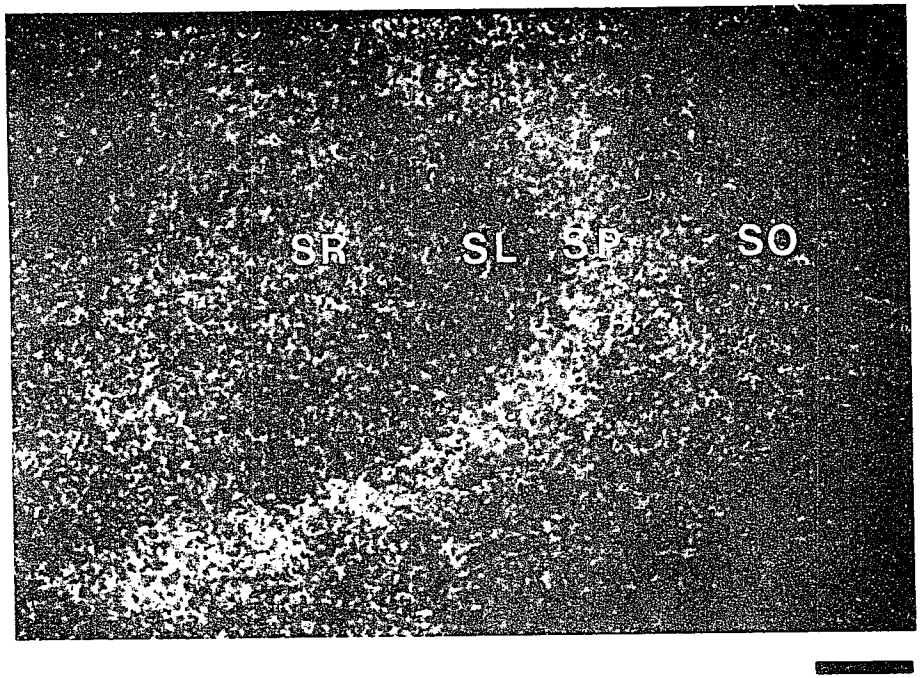

d.

Figure 7: Distribution of Rat BC1 RNA in Line #60 CA3 Field of the Hippocampal Formation.

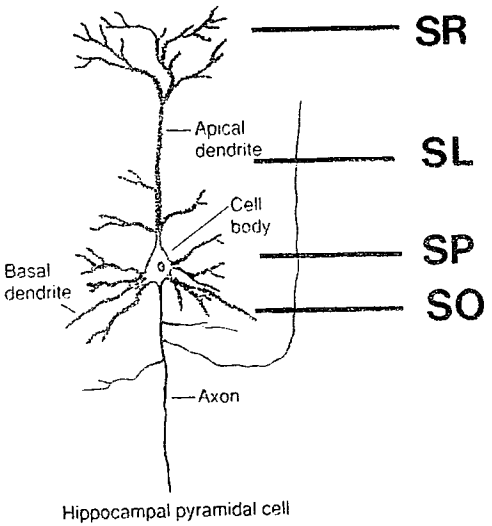
Panel a: rat BC1 RNA signal is observed in the layers of stratum pyramidale (SP), stratum radiatum (SR) and stratum oriens (SO) (less intensities) which contain the somata, the basal and the distal apical dendrites, respectively, of the CA3 pyramidal cells. Panel b: Diagramatic of CA3 pyramidal cells. The laminar structure of CA3 field of the hippocampal formation is shown: stratum oriens (SO), stratum pyramidale (SP), stratum lucidum (SL), stratum radiatum (SR) which is taken from "Principles of Neural Science".

Dark-feild optics, Nikon Microphot-FX. Scale bar, 30 μm.

a.


SR
SL
SP
SO

b.


SR
SL
SP
SO
Apical dendrite
Cell body
Basal dendrite
Axon
Hippocampal pyramidal cell

CHAPTER 4

THE COMMON SILENCER PROTEIN IS NOT ABLE TO BIND TO BC1 RNA GENE

It has been reported that the neuron-specific expression of the SCG10, human synapsin I and rat NaII channel genes appear to be controlled by a neural-restrictive silencer element (Mori et al., 1992, Kraner et al., 1992, Li et al., 1993). This element contains a sequence of 21 bp which is highly similar in the upstream regions of SCG10, human synapsin I and NaII genes. A neural-restrictive silencer binding factor (NRSBF), present in nuclear extracts from several different non-neuronal cell lines but not in extracts from neuronal cell lines, interacts with both the SCG10 and NaII silencers using a gel-shift assay. The data suggest that neuron-specific expression of SCG10 and NaII genes reflects, at least in part, the absence or inactivity of this protein. A similar region of homology was also present in the transcribed region of BC1 gene which partially overlaps with the internal promoter B box (Fig. 8). Thus, it was of interest to examine whether this element may play a similar functional role in BC1 RNA gene regulation. To identify whether the common silencer binding protein binds to BC1 RNA gene, electrophoretic mobility shift analysis was peformed.

Two BC1 gene probes have been generated as following: wild type of BC1 probe (BC1w) shares 70% homology with neural-restrictive silencer element; mutant of BC1 probe (BC1m) with a 8 bp change at the 5′ end of the probe. Meanwhile, S36 probe which contains the silencer element of SCG10 gene was used as positive control (Fig. 8) (see Method for detail). We performed electrophoretic mobility shift assay on nuclear extracts from Hela cells (kindly provided by Dr. D. Anderson at California Institute of Technology). The specificity of the gel shift with the S36 probe was established by a series of competition experiments. Specific competition was only obtained by using a 500-fold excess of S36 DNA, but not by using a similar excess of irrelevant CRE (c-AMP response element) or not even by 1,500-fold excess of BC1w and BC1m (Fig.9). In the parallel experiment, using BC1w as probe resulted in a weaker shift. The size of this complex was different from the shifting with S36 probe. Only with excess of BC1w and BC1m but not with 500-fold excess of S36 a specific competition was obtained. Our data suggest that it is likely that no common silencer protein binds to BC1w and BC1m. The specific binding of proteins to BC1w and BC1m might be due to the binding of TFIIIC to the internal B box. It was not surprising to see these results since similar region of sequence similarity was present in the promoter region of the rat synapsin I gene, which encode another neuron-specific protein. There is evidence showing that the silencer protein

can not bind to this gene either (personal communication of Dr. D. Anderson). It was thus concluded that, despite some sequence similarity, this sequence may not function as silencer in our rodent BC1 RNA gene.

THE ESTABLISHMENT OF RAT BC1-5' AND BC1-3' TRANSGENIC MICE LINES

The putative RNA polymerase III promoter region within the ID domain of the BC1 gene has remarkable sequence similarity to the consensus sequence of box A (all positions) and box B (all position except absence of one invariant A) in tRNA and other RNA polymerase III genes (Martignetti et al., in preparation). Therefore, tissue specific regulation of BC1 transcription may be governed by other cis-acting sequences located in the transcribed region or in the 5′ and /or 3′ flanking regions of the BC1 gene. The best example is the Bombyx mori silk gland $tRNA^{Ala}$ gene (Sprague et al., 1980) which is a tissue specific regulated and RNA polymerase III transcribed product, the control region encompasses both the coding region and 5′ as well as 3′ flanking region (Young et al., 1986).

Expression of the 1.4 kb rat BC1 gene in transgenic mice results in efficient and appropriate neuron specific expression, suggesting that the cis-acting regulatory

elements, subject to neuron-specific control, are located within the 1.4 kb rat BC1 gene (Chapter 3). To further localize the cis elements which are response for this regulation, the constructs with deletion of 5′ distal end (BC1-5′ or BC1-72) and 3′ distal flanking region (BC1-3′) of the 1.4 kb rat BC1 gene were generated (Fig. 10). The construct BC1-5′ contains the transcribed region plus 72 bp of 5′-flanking and 870 bp of 3′-flanking sequences which removes two octamer motif containing sequences. The BC-3′ construct deletes most 3′ flanking sequence of 1.4 kb BC1 gene which contains 429 bp upstream and 50 bp downstream from the BC1 gene.

Purified DNA was prepared and given to the Mount Sinai Transgenic Facility for microinjection. A total of 12 mice born from BC1-3′ construct. Thus far none were detected carrying transgene. However, two positive founders were identified from 15 mice born from BC1-5′ construct (Chapter 5). The positive founder mice were then crossed with wild-type C57BL/6 mice. Transgene BC1-5′ positive offsprings were sacrificed for analyzing BC1-5′ gene expression (see Chapter 5 for detail).

```
Consensus: TTCAGCACCACGGACAGTGCC
             T  A   G    G  CA
                T          A

Probes: S36 CAAAGCCATTTCAGCACCACGGAGAGTGCCTCTGC

        BC1w        TTTTCGGAGCTGAGGACCGAACCCAGGGCC

        BC1m        AGAAGCTTGCTGAGGACCGAACCCAGGGCC
```

Figure 8. The probes and competitors are indicated below the consensus sequence of silencer from SCG 10, neuron specific gene. These probes and competitors were used for Electrophoretic Mobility shift assays, which are shown in Figure 9. The region of maximum sequence identity is shown underline. The B box in BC1w and BC1m is indicated by double underline. S36 contains the silencer element of SCG 10 gene. BC1w, wild type BC1 gene, contains silencer homology element. BC1m, mutant of BC1w, contains 8 bp different from 5′ end of BC1w and, by analog to SCG 10, should not bind the putative silencer element.

Figure 9. Electrophoretic Mobility Shift assays were performed using HeLa nuclear extracts (kindly provided by Dr. D. Anderson). Probes used in the assays were either the restriction fragment containing two tandem copies of S36 (S36-dim) (Mori et al., 1992) or double stranded oligonucleotide BC1w containing the core silencer homology element (Figure 8). Competitors used in this study were non-radioactive S36, BC1w (wild type BC1), BC1m (mutant BC1) (Figure 8) and CRE (cAMP responsive element). The arrows indicate the complexes that are specifically competed.

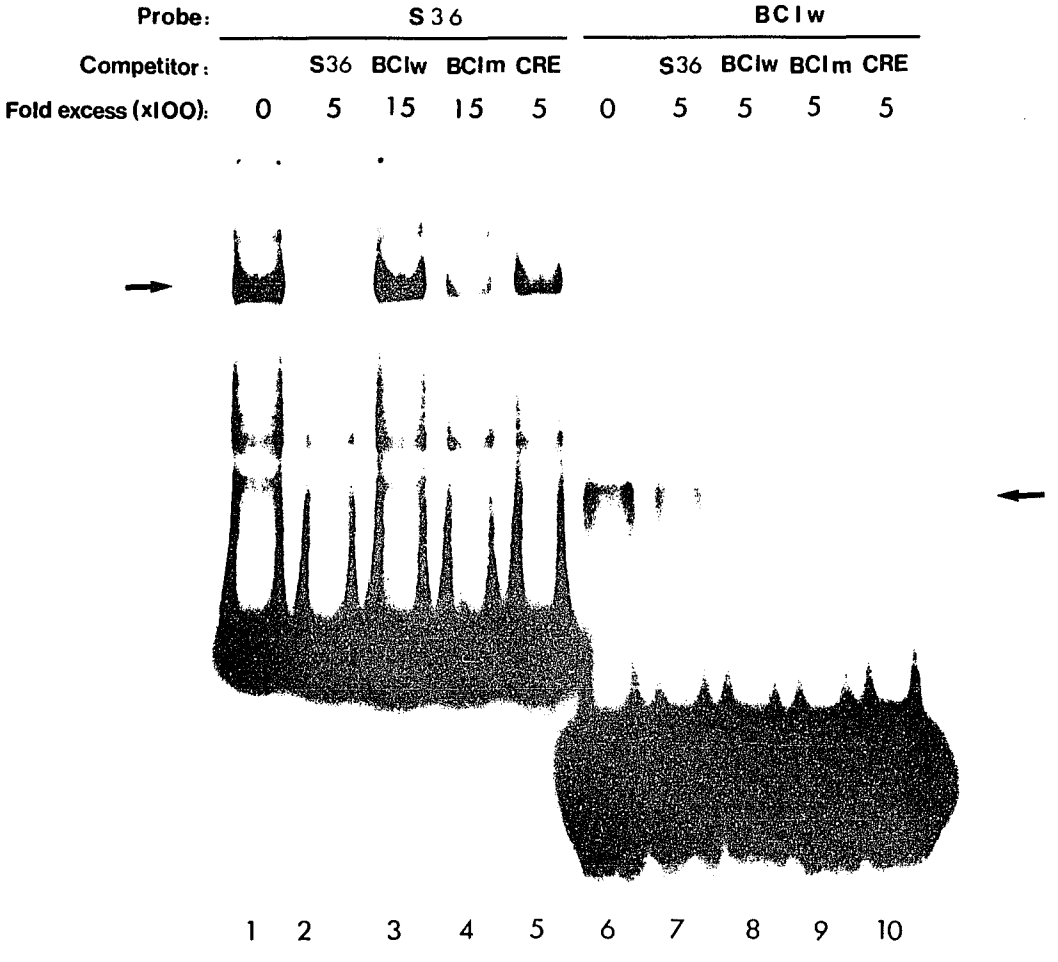
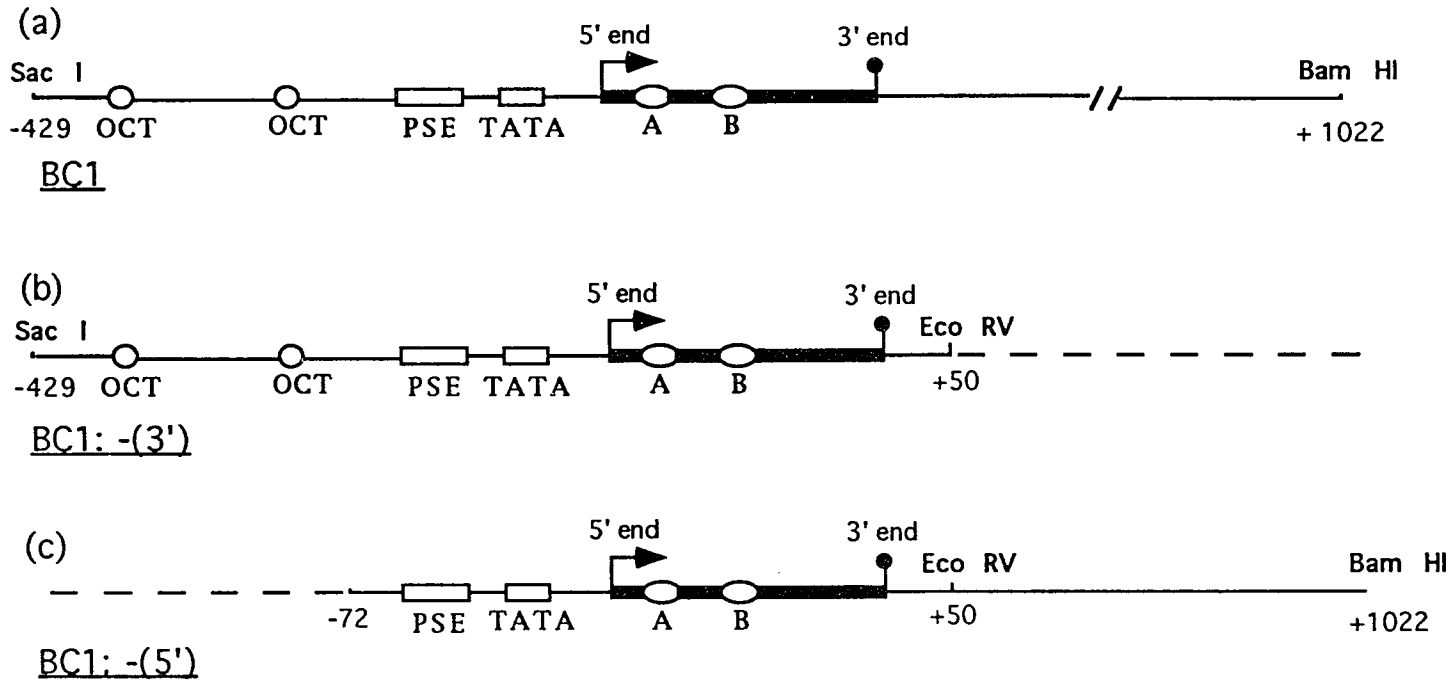

Probe:
S36
BCIw
Competitor:
S36
BCIw
BCIm
CRE
S36
BCIw
BCIm
CRE
Fold excess (x100):
0
5
15
15
5
0
5
5
5
5
1
2
3
4
5
6
7
8
9
10

Figure 10: Structure of rat BC1 RNA gene constructs.

a: Schematic diagram of the 1.4 kb rat BC1 gene which contains BC1 RNA transcribed region plus 429 bp of 5′-flanking and 870 bp of 3′-flanking sequences.

B: Schematic diagram of the BC1-3′ rat BC1 gene construct which contains BC1 RNA transcribed region plus 429 bp of 5′-flanking and 50 bp of 3′-flanking sequences.

C: Schematic diagram of the BC1-5′ (also named BC1-72 in chapter 5) construct which contains BC1 RNA transcribed region plus 72 bp of 5′-flanking and 870 bp 3′-flanking sequences.


(a)
5' end
3' end
Sac I
Bam HI
-429 OCT
OCT
PSE TATA
A
B
+ 1022
BC1
(b)
5' end
3' end
Sac I
Eco RV
-429 OCT
OCT
PSE TATA
A
B
+50
BC1: -(3')
(c)
5' end
3' end
Eco RV
Bam HI
-72
PSE TATA
A
B
+50
+1022
BC1: -(5')

CHAPTER 5

357-BP UPSTREAM OF PSE/TATA ELEMENTS OF RAT BC1 RNA GENE: A CANDIDATE FOR TRANSCRIPTIONAL ENHANCEMENT

ABSTRACT

Expression of the 1.4 kb rat BC1 gene in transgenic mice results in efficient and appropriate tissue-specific expression in the nervous system and testes but not in spleen, heart, liver, lung, kidney, lymphnode and other non-neuronal tissues. The expression is independent of the integration site. These results indicate that the cis-acting regulatory elements, subject to neuron-specific control, are located within the 1.4 kb fragment containing rat BC1 gene. To begin to identify which rat BC1 gene sequences are responsible for neuron-specific regulation, additional transgenic mouse lines containing a deletion at the 5′ distal end of the 1.4 kb rat BC1 gene (BC1-72) have been characterized. Deletion of 357 bp including two octamer motifs has no effect on the tissue specificity. However, the average level of expression per transgene copy is about 5% as much of the endogenous mouse BC1 RNA and about 5 times less than with the 1.4 kb rat BC1 gene. Furthermore, the integration site-independence disappeared. We conclude that the sequences of rat BC1 RNA gene containing two octamer motifs are not essential for the neural specific expression of BC1 RNA. However, elements including the octamers may be candidates for transcriptional enhancement. In addition, integration site-independent expression of BC1 transgene requires the presence of the 357 bp fragment of the 5′ flanking sequence or a portion thereof.

INTRODUCTION

Tissue-specific expression of eukaryotic genes is conceptualized as the result of a complex set of DNA-protein and protein-protein interactions (He and Rosenfeld., 1991; Johnson and McKnight., 1989, Struhl 1991). Both positively and negatively acting DNA elements appear to be involved, with the balance determining the absolute level of transcription in a given cell type (Mandel and McKinnon, 1993). As a step toward understanding the mechanisms regulating neuron-specific gene expression, it is necessary to identify and analyze the interplay between specific transcription factors and their corresponding cis-acting DNA sequence. Rodent BC1 RNA is a brain specific cytoplasmic small RNA which is the first example of a RNA polymerase III transcription product with neuron specific and developmental regulation (Sutcliffe et al., 1982, 1984). It is an interesting model for analyzing the regulatory sequences of neuron-specific RNA polymerase III genes.

Data from our previous studies, transgenic mice generated with the cloned 1.4 kb rat BC1 gene expressed the transgene efficiently and in appropriate tissues even at subcellular levels. In addition, the expression was integration-site independent (Chen et al., in preparation). To extend these studies, we have investigated in transgenic mice the

dependence of BC1 gene expression on the distal 5′-flanking sequence (357 bp containing two octamer motifs), although our *in vitro* transcriptional studies had shown that the two octamer containing sequences were not necessary for efficient transcription of BC1 RNA. (Martignetti and Brosius, in preparation). It has been known that the octamer element acts as a positive regulator of many cellular gene promoters, which include those of the small nuclear RNAs, the histone H2B gene, and immunoglobulin genes (reviewed by Falkner et al., 1986). It also has been reported that the octamer motif is capable of mediating the repression of octamer-containing cellular promoters in neuronal cells (Kemp et al., 1990, Lillycrop et al., 1994). Those differences in the pattern of gene activity mediated by the octamer element is believed to be due to differences in its relationship to other elements in the promoter that affect its recognition by different octamer binding proteins (Tanaka et al., 1988). Several octamer binding proteins have been identified in different cell types including the brain (He et al., 1989, Lillycrop et al., 1994) and embryonal carcinoma cells (Lenardo et al., 1989; Scholer et al., 1989) in addition to the constitutively expressed octamer binding protein OTF-1, OTF-2.

In this report, we show that the rat BC1-72 deletion construct was introduced into the mouse genome. The expression of transgene BC1 RNA was compared with that of mice containing

the longer 1.4 kb construct. The results of these studies indicate that neuron-specific cis-acting sequences are present within the 1 kb BC1 gene fragment. However, one or both octamer motifs and/or other unidentified elements within this deleted region are necessary for the efficient and integration-site independent expression of rat BC1 gene in transgenic mice.

METHODS

Genomic DNA was extracted from the tails of 15 candidate transgenic mice, and from rat and a non-transgenic mouse used as positive and negative controls, respectively. Approximately 10 ng DNA from each of them were then PCR analyzed in 30 cycles (denaturation for 30 sec at 94°C, annealing for 1 min at 58°C, extension for 2 min at 75°C; initial denaturation was for 4 min at 94°C, final extension was for 10 min at 75°C), using the rat BC1 gene specific primers 5′-TGGTGGGTGGGGTCGGGCCAA-3′ (WCR001) and 5′-AAACAAGGTAACTGGCACACACAACCTTT-3′ (sense). The products were size fractionated on a 1% agarose gel (Fig. 12). Transgene copy number was determined by dot blot hybridization (Palmiter et al., 1983) with the ^{32}P-labelled oligonucleotide probe corresponding to the 3′-unique region of BC1 RNA followed by quantitation using phosphorimage scanning from several experiments.

Preparation and analysis of RNA

Positive offspring from founders #9 and #12 carrying a BC1-72 transgene were sacrificed and fresh tissues were removed and immediately frozen in liquid nitrogen. Total RNA was isolated by homogenization in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion. RNA (10μg) was fractionated on 1.8% agarose-formaldehyde gels for Northern blot analysis (Maniatis et al., 1982). The rat and mouse BC1 RNA specific oligonucleotide probes, as described previously (Chen et al., in preparation), were labelled with [γ-^{32}P] ATP by T4 polynucleotide kinase. Hybridization was performed at 37°C in 1M NaCl, 0.5M Tris-HCl (pH 7.5), 5X Denhardt's reagent, 1% SDS and 0.1mg/ml yeast tRNA . The membranes were then washed three times at 45°C in 2X SSC and 0.1% SDS for 30 min. The expression levels of BC1-72 transgene were then quantitated by phosphorimage scanning from several experiments.

In Situ hybridization

Fresh brain tissue from positive offspring were quick frozen using liquid nitrogen, embedded in Tissue-Tek OCT embedding medium, and then sectioned in a Bright Microtome Cryostat at 10 μm thickness. Sections were collected on gelatin and poly-L-lysine coated microscope slides.

A transcription vector (pMK1) containing a sequence that represent the 60 nucleotides of 3' most region of BC1 RNA was

subcloned into pBluescript KS(+) vector (Tiedge et al., 1991). ^{35}S- labelled RNA probes ("sense" and "antisense" strands) were transcribed from prelinearized templates using T3 or T7 RNA polymerase (BRL protocol).

In situ hybridization experiments were performed as previously described (Tiedge, 1991). This protocol uses UV- light as cross linking agent to improve signal-to-background ratios.

RESULTS

Neuronal Specific Expression Of BC1-72 Transgene

Previous studies indicated that each of three lines of the 1.4 kb rat BC1 transgenic mice expressed the rat BC1 RNA in appropriate tissue, at levels of about 25% as much of endogenous mouse BC1 RNA. To extend these studies, additional transgenic mice were prepared from deletions of the 5′ flanking portions of the 1.4 kb rat BC1 gene. This deletion (375 bp) removes two octamer containing sequence at its 5′ end (Fig. 11). Total DNA prepared from the tails of 15 candidate mice were analyzed by PCR for presence of transgene using primers specific to rat BC1 gene (see methods for detail). DNA from rat and wild-type C57BL/6 mouse were used as positive and negative controls, respectively. Two positive founders (#9 and #12) of the BC1-72 transgenic mouse lines were identified which was shown on Figure 2. Liver DNA from positive animals were then analyzed by dot blot to determine

transgene copy number. The two founder mice (#9 and #12) carried about 4 copies each of the transgene (data not shown).

The expression pattern of the BC1-72 transgene was determined by Northern blot analysis with oligonucleotide probes specific to either transgene-encoded rat BC1 RNA or endogenous mouse BC1 RNA as described previously (Chen et al., in preparation). One of the two lines carrying the 357 bp deletion construct expressed transgenic BC1 RNA with the same tissue specificity as found for the 1.4 kb BC1 transgenic mice, although there are differences in the level of transgene RNA among these lines (Fig. 13 and 14). Rat BC1 RNA was detected in #9 transgenic mouse brain but not in liver and lung. As endogenous mouse BC1 RNA, low levels of transgenic BC1 RNA signal was also detected in testes. A cDNA probe for β-actin was included as control for integrity and amount of RNA on the blots (Fig.13). Transgene RNA was not present in other non-neuronal tissues including kidney, heart, spleen and lymphnode (data not shown). These results indicated that the expression of the BC1-72 transgene was brain specific with a similar distribution pattern as endogenous mouse BC1 RNA. The sequences containing two octamers and/or other unidentified elements were not required for neural specific regulation.

The subcellular distribution of the BC1-72 transgenic RNA was

determined by in situ hybridization as described previously (Chen et al., in preparation). In comparison with non-transgenic mouse brain, there was no detectable ectopic expression in BC1-72 transgenic mouse brain (data not shown). These anatomical data with the observed labeling pattern indicate that all necessary elements for the cell type specific expression are located on the 1 kb BC1-72 rat BC1 gene.

A 357-bp 5′ distal sequence of BC1 gene needed for efficient, integration site-independent expression

In contrast to our results obtained with the 1.4 kb rat BC1 transgenic mice, deletion of the 5′-flanking sequences resulted in a dramatic reduction of rat BC1 RNA expression levels in transgenic mouse brain. Estimates of brain RNA levels of the BC1-72 mice are compared with those of the 1.4 kb BC1 mice and summarized in figure 4 and Table 2. As there are two copies of the mouse BC1 gene per diploid genome, the levels of rat and mouse BC1 RNA were divided by their respective gene copy numbers to make a direct comparison of expression.

Figure 14 and Table 2 demonstrate that all three transgenic mice containing 1.4 kb rat BC1 gene expressed the transgene at levels ranged from about 125-350% of endogenous mouse BC1 RNA. The average level of expression per gene copy was about 25% of

endogenous mouse BC1 RNA. When the rat BC1 gene without 357 bp 5′-flanking region was analyzed in transgenic mice, only one of the two transgenic lines expressed the rat BC1 RNA and the levels ranged from 5-15% of endogenous mouse BC1 RNA. When these values were corrected for transgene copy number, the average level of expression per gene copy was 5% of endogenous mouse BC1 RNA (Table 2). Thus, deletion of the 5′ end of the 1.4 kb BC1 gene results in more than a 5-fold decrease with respect to transgene expression in mouse brain. In addition, all of the transgenic animals that contained the 357 bp fragment expressed rat BC1 RNA, while one line that did not contain these sequences was expressed at relatively low levels and the other line did not express the transgene at all. These data suggest that elements in the 5′ most 357 bp of the 1.4 kb fragment may be involved in enhancer activity and that the same and/or other sequences confer position independent expression.

DISCUSSION

Tissue specific expression of the rat BC1 gene in transgenic mice

A construct containing a 357 bp deletion at the 5′ end of the 1.4 kb rat BC1 gene was introduced into the genome of two mice. Transgene expression was in the appropriate tissues at detectable levels in one of the two lines and did not show

ectopic expression even at the subcellular level. This expression pattern is similar to the one for the 1.4 kb transgenic mouse and for the endogenous mouse BC1 gene. We conclude that if there are regulatory elements essential for neuronal specific expression of the rat BC1 RNA gene, they must reside within the BC1-72 (containing transcribed region plus 72 bp of 5′-flanking and 870 bp of 3′-flanking sequences), i.e. 1 kb fragment of BC1 gene. The 357 bp containing two octamers and possibly other sequences are not essential for the tissue specific regulation of BC1 gene expression.

An unexpected finding, beyond the scope of this paper, was that the transcription levels of endogenous 7SL RNA was altered in neural tissue of transgenic mice. The reduction of 7SL RNA was observed in both 1.4 kb rat BC1 and BC1-72 transgenic mouse brain (Fig. 13 and 14). However in non-neuronal tissues including liver, lung, kidney, spleen and heart the transcription of 7SL RNA remains at similar levels as for non-transgenic mouse (data not shown). No similar reduction in other polymerase III transcripts such as 7SK and 4.5S RNA was observed (Fig. 13 and 14). These data imply that the reduction might be related to some factors, essential for transcription or processing of both BC1 and 7SL RNA (but not 7SK of 4.5S RNAs). Such factors may be decreased during overexpression of BC1 RNA in transgenic mouse brain due to

competition. Therefore the transcription or stability of 7SL RNA was reduced. The possibility of this reduction due to the integration of transgenes into the mouse genome interfering with the host 7SL RNA locus could be ruled out since it was observed in all transgenic mice and in brain only. Further experiments are necessary to explain this finding.

Role of the 357 bp 5′ sequence on rat BC1 RNA expression

In most transgenic experiments, the integration site of foreign genes usually accounts for significant differences in the level of expression as well as the tissue specificity of expression (Palmiter and Brinster, 1986). These differences are often due to potential cis-acting regulatory elements flanking the site of integration (Al-shawi et al., 1990). Only a few genes are expressed independently of their site of integration (Grosveld et al., 1987, Bonifer et al., 1990, Abe et al., 1990, Chamberlain et al., 1991, Aronow et al., 1992). In the case of the beta-globin gene, four erythroid-specific, DNase I super-hypersensitive sites (HS) are located 11 to 18 kb upstream of the human β-globin gene family. These sequences are critical for high-level and tissue-specific expression of human β-globin genes and, therefore, have been designated the Locus Control Region (LCR). Felsenfeld (1992) recently suggested that a primary role of the β-globin LCR is to inhibit nucleosome assembly on globin gene promoters during DNA replication. LCR binding proteins form stable interactions

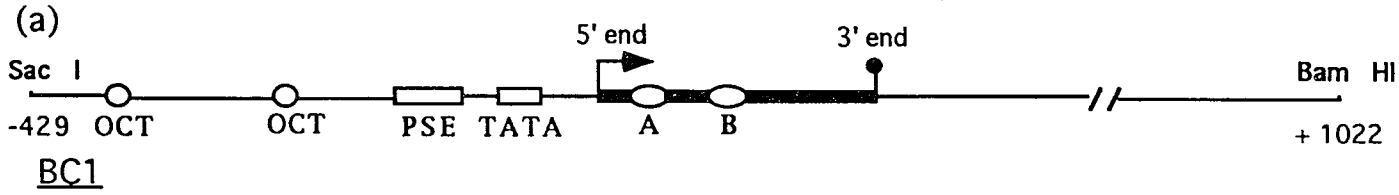
with promoter binding factors and keep the promoter in an open conformation specifically in erythroid cells. Similarly in the lysozyme gene, the A element that was associated with nuclear matrix attachment activity conferred both integration site-independent and copy number-dependent expression of a CAT reporter gene construct in cell lines.

In this study only one of two animals without two octamer containing sequence expressed the transgene which indicted that the construct with 5′ deletion is more sensitive to chromosomal integration site. In contrast, three of three animals carrying the 1.4 kb construct (without 5′ deletion) expressed transgene BC1 RNA in the appropriate tissue. These results suggest that the 357 bp at the 5′ end of the 1.4 kb rat BC1 gene construct is able to activate expression regardless of the site of transgene integration. Furthermore, the average levels of expression per gene copy were high for all three 1.4 kb BC1 mice compared to the 5′ deletion BC1-72 mice. The rat BC1 construct was expressed at an average level of 25% of endogenous mouse BC1 per gene copy. The average level of expression per gene copy for BC1-72 construct was only 5% of endogenous mouse BC1. This average level of expression is five times lower than the full-size 1.4 kb construct containing two octamer motifs. Since only one transgenic line with BC1-72 construct expressed BC1 RNA, additional transgenic animals generated with the same

construct are necessary to confirm these studies.

In summary, the 357 bp 5′ sequence containing two octamers stimulated the average level of BC1 gene expression at least five fold more than that without these sequences. The octamers and/or other cis-acting elements may be candidates for transcriptional enhancers. In addition, the 357 bp two octamer containing sequence increases the fraction of transgenic animal that express the rat BC1 gene. Possibly, this sequences ensures that the transgene will be in an open chromatin domain regardless of the site of integration. Caterina et al., 1994 have reported that multiple protein binding sites (SP-1, NF-E2, GATA-1 or USF) within 5′ HS2 of LCR are sufficient to prevent chromosomal position effects that inhibit transgene expression. Deletion of SP-1, GATA-1 and USF binding sites reduce human β-globin gene expression from 41.5% per gene copy to approximately 10% per gene copy in transgenic mice. Deletion of the duplicated NF-E2 sites reduced expression to 2% per gene copy. It is possible that the two octamer binding sequences within 357 bp play the similar role as one of these multiple protein binding sites found on human β-globin gene. Deletion and/or alteration of the one or both octamers will be necessary to confirm this notion. It also still remains to be determined if and what other elements within the 5′ distal 357-bp of BC1 gene are necessary and sufficient for enhancer activity.

Figure 11: Panel a: Schematic diagram of the 1.4 kb rat BC1 gene which contains both upstream and coding region promoter elements as indicated. Panel b: Schematic diagram of the rat BC1-72 gene, 357 bp 5′ distal region deletion of the 1.4 kb construct, used for generate the transgenic mice. The symbols are as noted above.


(a)
5' end
3' end
Sac I
Bam HI
-429 OCT
OCT
PSE TATA
A
B
+ 1022
BC1

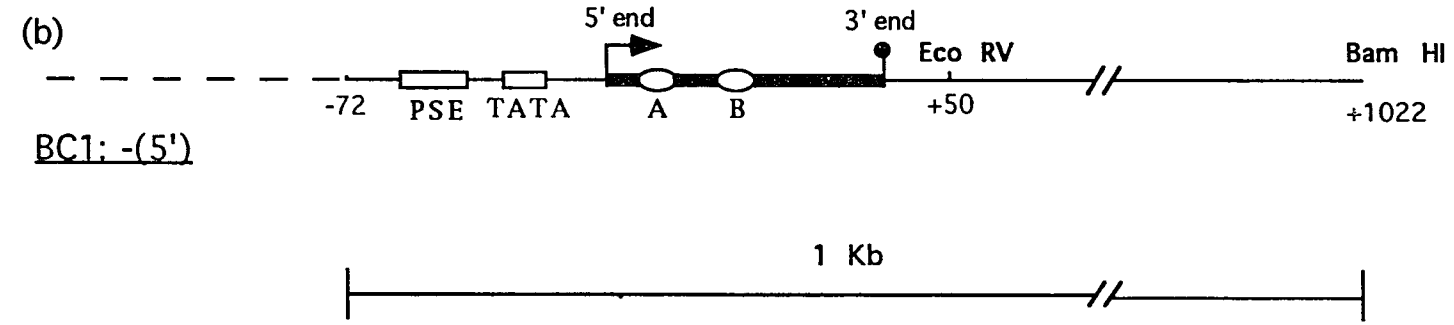

(b)
5' end
3' end
Eco RV
Bam HI
-72
PSE TATA
A
B
+50
+1022
BC1: -(5')
1 Kb

Figure 12: PCR Analysis of the DNA from 15 Candidate Mice. Approximately 10 ng of each DNA sample, extracted from the tails of 15 candidate mice (1-15), non-transgenic mouse (Mo), and rat (R), respectively, was PCR analyzed for presence of the transgene using specific primers to rat BC1 gene (from downstream region position +123 to +536 of the rat BC1 gene), as described in methods. The PCR products were then separated on a 1% agarose gel. The rat BC1 RNA gene corresponds to the 413 bp fragment seen in rat sample (R), positive control, and two BC1 positive transgenic mice ($^{\#}$9 and $^{\#}$12) but not in nontransgenic mouse (Mo), negative control. The positions of size standards are indicated to the left.

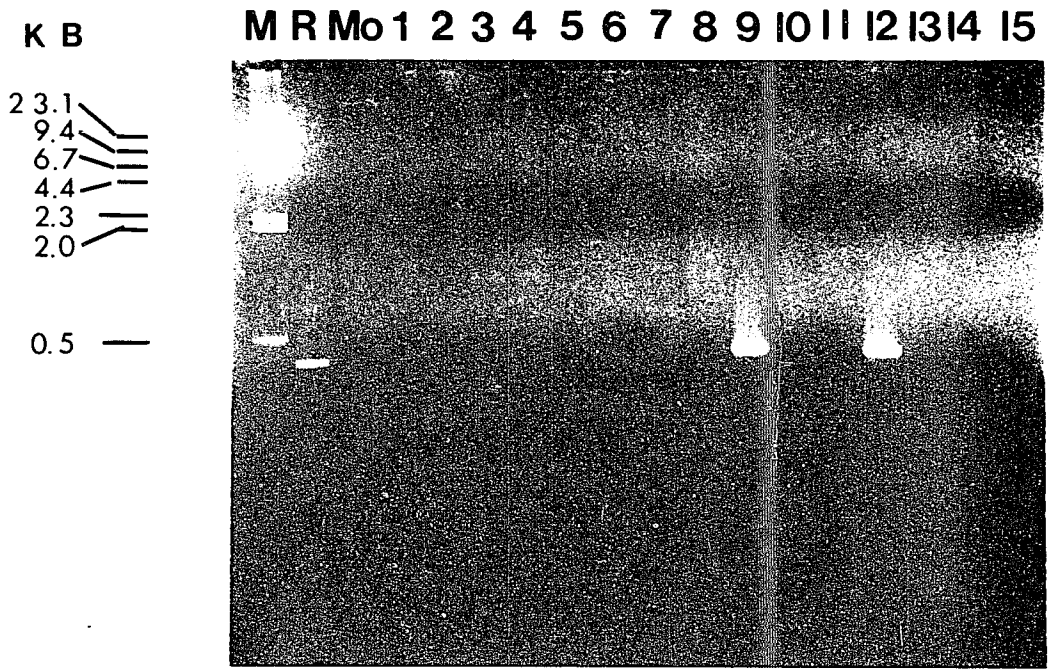

K B
23.1
9.4
6.7
4.4
2.3
2.0
0.5
M R Mo 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 13: Northern Blot Analysis of BC1-72 Transgene Expression in Transgenic Mice.

Total RNA (10 μg per lane) was extracted from transgenic mouse brain (B), liver (L), lung (Lu), testis (T), nontransgenic mouse brain (MB) and rat brain (RB), and was separated on a 1.8% agarose-formaldehyde gel. It was then blotted onto a nylon membrane, and hybridized with ^{32}P-labelled rat BC1 (panel a), mouse BC1 (panel b), rat β-actin (panel c), human 7SL (panel d) and rat 7SK (panel e) probes, respectively.

Using mouse BC1 probe, a positive signal was detected in mouse brain, #9 and #12 brains. A weak signal was also present in both transgenic mice testes. No signal was detected in rat brain, both #9 and #12 livers and lungs. Using rat BC1 probe, a positive signal was detected in rat brain and #9 brain but not in mouse brain, #12 brain, and both livers and lungs. β-Actin, 7SL and 7SK probes hybridized to all samples.

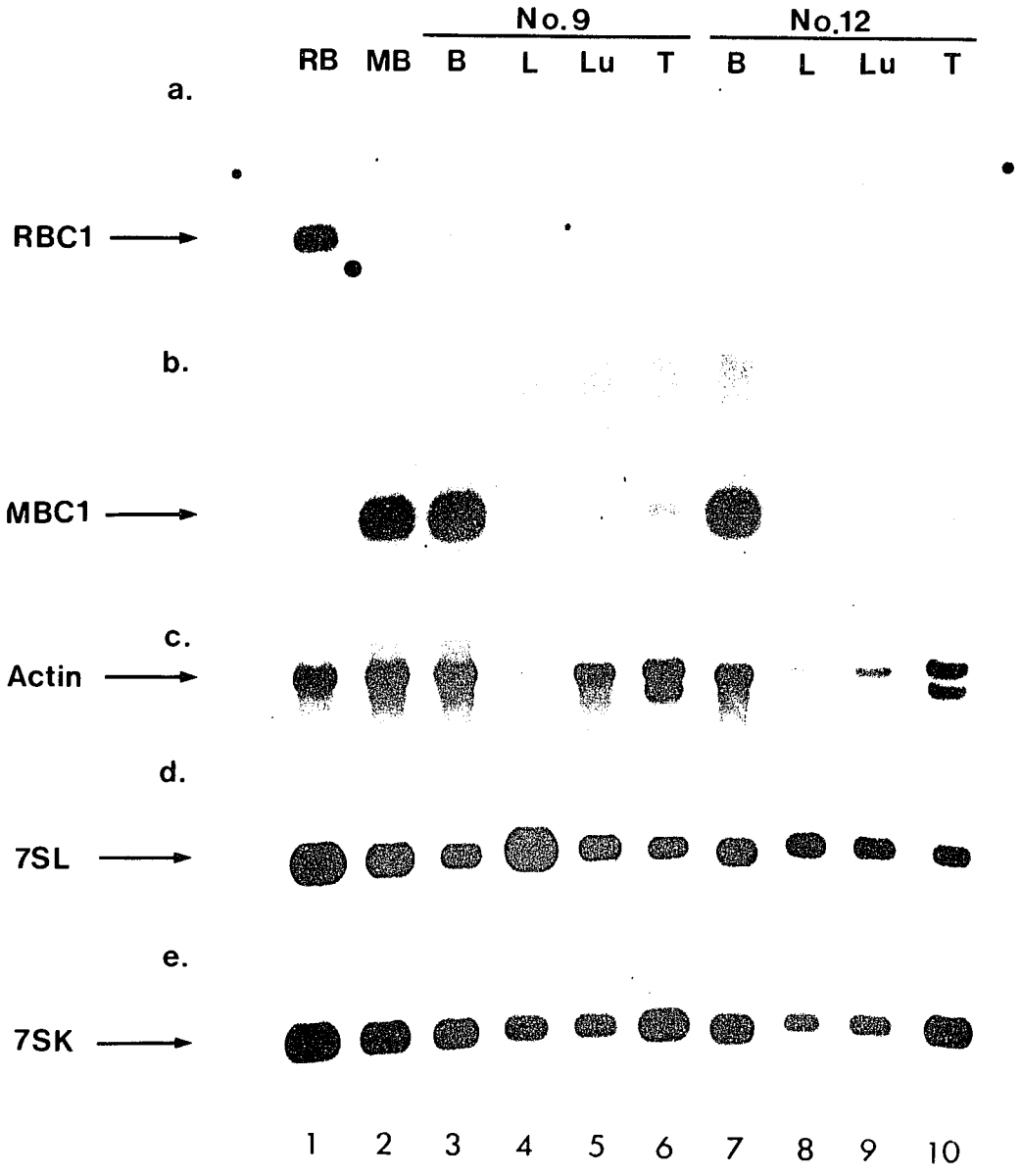

No.9
No.12
RB MB B L Lu T B L Lu T
a.
RBC1
b.
MBC1
c.
Actin
d.
7SL
e.
7SK
1 2 3 4 5 6 7 8 9 10

Figure 14: Expression levels of transgene RNA from rat BC1 and BC1-72 mice.

Total RNA (10 μg per lane) extracted from rat brain (RB), nontransgenic mouse brain (MB) and from ($^{\#}$54, $^{\#}$59 and $^{\#}$60) 1.4 kb rat BC1 transgenic mouse brain (TMB) as well as ($^{\#}$9 and $^{\#}$12) BC1-5′ transgenic mouse brain (TMB) was analyzed by Northern hybridization usin rat BC1 (panel a), mouse BC1 (panel b), actin (panel c), 7SL (panel d), 7SK (panel e) and 4.5S (panel f) probes respectively.

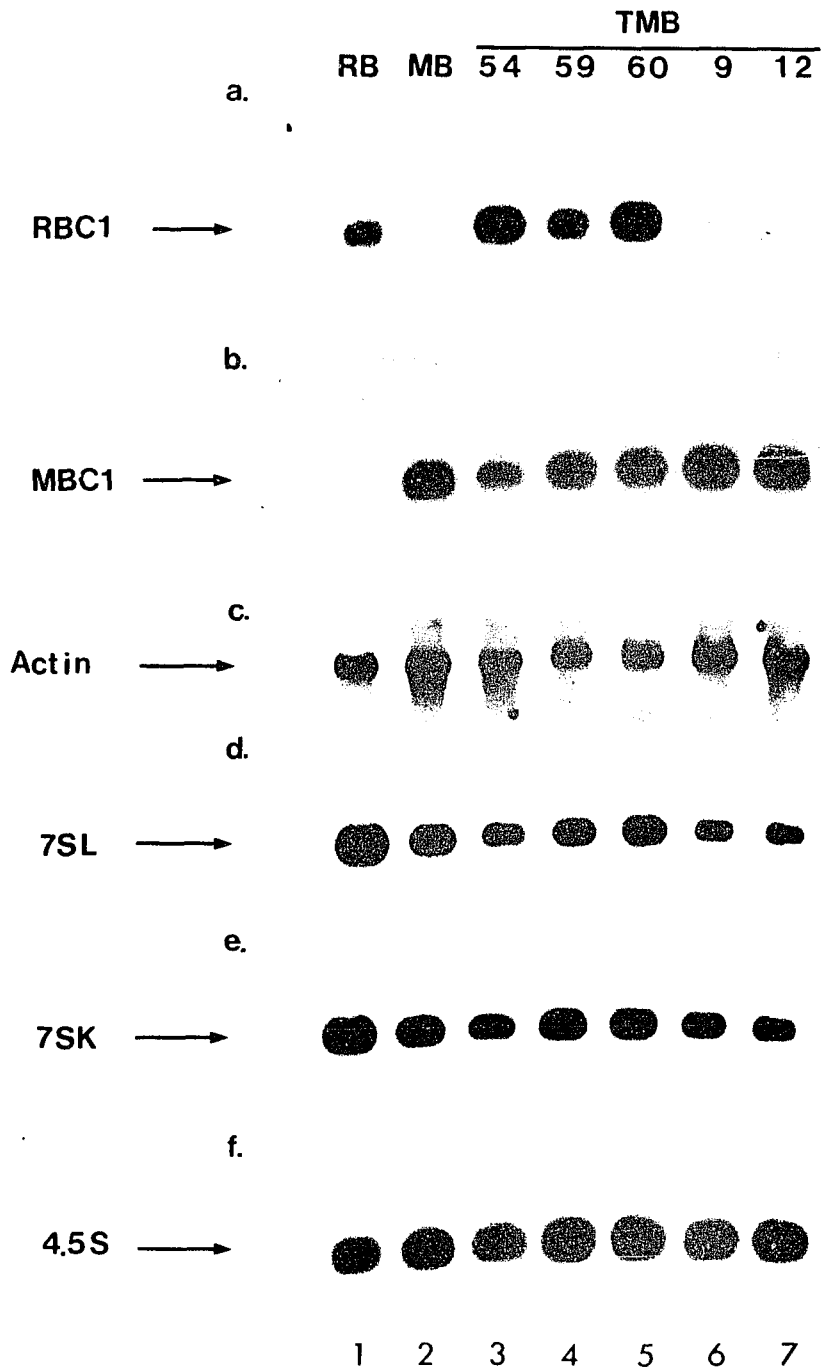

TMB
RB MB 54 59 60 9 12
a.
RBC1
b.
MBC1
c.
Actin
d.
7SL
e.
7SK
f.
4.5S
1 2 3 4 5 6 7

Table 2: Summary of rat BC1 transgene expression.

Transgene	Fraction expressor	Transgene copies[a]	Percent endogenous mouse BC1 RNA[b]	Percent expression pergene copy[c]
BC1	3/3	14-20	125-350	18-35
BC1-72	1/2	4	5-15	2-8

a. The number of transgene copies was determined as described in methods from several experiments. The values were normalized to wild-type C57BL/6 as 2 (two copies of the mouse BC1 gene per diploid genome).

b. The levels of transgene expression were analyzed by Northern blot hybridization followed by quantitation using phosphorimage scanning. The values were normalized to wild-type C57BL/6 as 1.

c. The values of percent expression per gene copy were calculated assuming two mouse BC1 genes per cell. (rBC1 RNA/r BC1 gene)/(mBC1 RNA/mBC1 gene)

CHAPTER 6

INDUCTION OF RODENT BC1 RNA IN MOUSE TUMOR TISSUES

ABSTRACT

Rodent BC1 RNA is the first identified RNA polymerase III transcribed cytoplasmic small RNA under neuronal specific and developmental regulation. It has been reported that BC1 RNA is deregulated in immortalized cell lines (McKinnon et al., 1986). In this study, we try to determine whether the tissue-specific expression of BC1 RNA gene is altered in tumor tissues using Northern blot and in situ hybridization analysis. Oncogene and chemical carcinogens induced tumor mouse models were used in the study. BC1 RNA was selectively expressed in tumor cells, although the levels were variable from case to case, but not in the corresponding normal tissues and intratumor stroma cells. The study first demonstrated that BC1 RNA was deregulated in mouse tumor tissues.

INTRODUCTION

A short interspersed repetitive DNA element (SINE), the ID sequence, is about 75 nucleotides long and is distributed 10^5 times in the rodent genome. This SINE contains internal RNA polymerase III promoter box A and B (Sutcliffe et al., 1982; 1984). ID sequences are mainly if not exclusively transcribed by RNA polymerase II. Copies of the ID elements are found within intervening sequences or in untranslated regions of polymerase II genes, and are hence transcribed by RNA polymerase II as part of the large primary transcrips in

a variety of tissues (Sutcliffe et al., 1984, Owens et al., 1985). BC1 RNA, one of the founders of ID elements is transcribed by RNA polymerase III and generates a neuronal specific cytoplasmic small RNA. Even though a few ID elements may be transcribed by polymerase III, there are no known examples. Previous studies have demonstrated that the ID family of repetitive elements are regulated during developmental stages and oncogenic transformation (Herget 1986, Glaichenhaus and Cuzin 1987). The ID repeat elements are found to be accumulated following growth-factor-induced transition of normal (FR3T3) rat fibroblasts from a quiescent to a proliferative state and also following transformation by virus and by oncogenes (Glaichenhaus and Cuzin 1987). It has been suggested that the ID repetitive sequences play a regulatory role in growth- and transformation-dependent gene expression (Herget 1986; Glaichenhaus and Cuzin 1987).

Rodent BC1 RNA, which is a master or source for at least some of the ID repetitive elements (Kim et al., 1994), was discovered by Sutcliffe et al. (1982, 1984). It is transcribed by RNA polymerase III, developmentally regulated and prevalently expressed in nervous tissue. However undifferentiated germ cells in male and female gonads are also found to express BC1 RNA at detectable levels. It has been reported that rodent BC1 as well as primate BC200 RNAs, an analogous of BC1 RNA, are expressed in immortalized cell lines

(Mckinnon et al., 1986, Watson and Sutcliffe, 1987). Our recent studies show that BC200 RNA is deregulated in certain tumor tissues and might play a role in the pathagenesis of those tumors under some circumstances (Chen et al., in preparation). We now report that BC1 RNA is also deregulated in mouse tumor tissues. This small RNA is selectively accumulated in malignent cells but not in the adjacent normal tissues and intratumor stroma cells.

MATERIAL AND METHODS

Three different mouse tumor samples were obtained from Dr, Srivastava (Department of Pharmacology, Mount Sinai Hospital).Fibrosarcoma of skin and adenocarcinoma of colon were induced by local innoculation of methlycholanthrene-induced fibrosarcoma cell line and cycasin-induced adenocarcinoma cell line, respectively (Weisburger, 1971). Breast carcinoma was induced by ras-oncogene(Andres et al., 1987).

Preparation and analysis of RNA

Mouse tumor tissues and their adjacent normal tissues were removed and immediately frozen in liquid nitrogen. Total RNA was isolated by homogenization in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion. RNA (10 μg) was fractionated on 1.8% agarose-formaldehyde gels, transferred onto a GeneScreen membrane, immobilized by UV illumination and hybridized to oligonucleotide probe HT005. The probe is

complementary to the 3′ unique BC1 RNA sequence 5′-AAAGGTTGTGTGTGCCAGTTACCTTG TTTTTTTTTGGTCTTTTTGTTATTTTGTCTTTTT-3′ and labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The same blots were then rehybridized to a rat β-actin cDNA probe. Hybridization was performed at 42°C for the BC1 probe, at 60°C for the rat β-actin probe, in 1M NaCl, 0.5M Tris-HCl (pH 7.5), 5X Denhardt's reagent, 1% SDS and 0.1mg/ml yeast tRNA . The membranes were then washed three times at 55°C in 0.5X SSC and 0.1% SDS for 30 min for the BC1 probe, and at 65°C in 0.1X SSC and 0.1% SDS for 30 min for the rat β-actin probe.

In Situ hybridization

Fresh tissues from mouse tumors and their adjacent normal tissues were quick frozen using liquid nitrogen, embedded in Tissue Tek OCT embedding medium, and then sectioned in a Bright Microtome Cryostat at 10 μm thickness. Sections were collected on gelatin and poly-L-lysine coated microscope slides.

The probe used to detect mouse BC1 RNA in the in situ hybridization experiment is corresponding to the 60 nucleotides of 3′ unique region of BC1 RNA and was subcloned into pBluescript KS(+) transcription vectors resulting in PMK1 (Tiedge et al., 1991). ^{35}S- labelled RNA probes ("sense" and "antisense" strands) were transcribed from prelinearized templates using T3 or T7 RNA polymerase (BRL protocol).

In situ hybridization experiments were performed as previously described (Tiedge, 1991). This protocol uses UV- light as cross linking agent to improve signal-to-background ratios.

RESULTS AND DISCUSSION

Expression of BC1 RNA in three different mouse tumor samples was examined by Northern blot analysis. Total RNA (10 μg) from three different tumor tissues and their corresponding normal tissues were hybridized to ^{32}P labeled probe HT005 complementary to the 3′ unique region of BC1 RNA (see methods for detail). RNA from rat brain was used as a positive control. A significant amount of BC1 RNA was observed in all three tumor tissues in comparison to the normal tissues (Fig. 15a). The same blot was rehybridized to a β-actin probe to monitor quantity and integrity of the RNA preparations (Fig. 15b). Our data suggest that BC1 RNA is deregulated in those tumor tissues.

We then analyzed the distribution of BC1 RNA in mouse tumor tissues by in situ hybridization. Although the staining pattern is different from case to case, it varied from scattered staining of a few positive cells to extensive staining of most of the malignant lesion, BC1 RNA was restricted to tumor cells but not found in intratumor stroma cells(Fig. 16 and 17). In cutaneous fibrosarcoma of the

mouse, BC1 RNA signal was present in most of the tumor cells in the dermis where it was located in the cytoplasm. The normal epidermis including rete ridges were negative for BC1 RNA (Fig. 16A and B). A similar staining pattern was observed in colon adenocarcinoma (Fig. 16C and D). In ras-oncogene induced mouse breast carcinoma, a heterogeneous staining pattern was present within the same section. For instance, BC1 RNA staining was observed only in a few tumor cells in one region whereas it was expressed in most of the tumor cells in other region (Fig. 17). The sense control probe failed to produce specific labeling in any of the examined tissues (Fig. 16E and F). In situ hybridization studies further confirmed that the origin of transcription of this small RNA was restricted to tumor cells.

In this paper, we have analyzed the induction of BC1 RNA gene expression in ras-oncogene and chemical carcinogen induced mouse tumor tissues. BC1 RNA is accumulated in tumor cells but not in adjacent normal tissues and the cells within the stroma. The expression pattern is similar to that of primate BC200 RNA in human tumor tissue where the malignant cells are the predominant site for transcription of both BC1 and BC200 RNAs. Since in these studies fibrosarcoma of skin and adenocarcinoma of colon are induced by local innoculation with cells from a methylcholanthrene-induced fibrosarcoma line and cycasin-induced carcinoma line, respectively. We can not rule

out the possibility that the positive signal in both tumor tissues might be due to overgrowth of the BC1 positive cell lines in the host. There is no evidence that local innoculation of malignant cell lines could cause host cell malignance. Transgenic mice have been used as models for the molecular analysis of tumorigenesis for almost a decade. Overexpression of oncogenes, such as ras, myc and neu, in mammary tissue results in development of breast cancer (Cardiff et al., 1991). Mouse breast tumor, used in these studies, is induced by ras-oncogene and is a primary tumor which avoids the problems mentioned above. Therefore it is a better model and the data are more convincing.

BC1 RNA is neural-specific RNA pol III transcribed small RNA (Sutcliffe et al., 1982;1984). In addition to general pol III transcription factors (TFIII B and TFIII C), the presence of specific protein activator(s) or absence of silencer-binding proteins in neurons may contribute to it's neural-specific expression. It is possible that during tumorigenesis, those factors could be directly or indirectly under- or over-expressed or deregulated in cancer tissues which could activate or deinhibit the BC1 RNA gene promoter. Alternatively, the stability of this RNA might be enhanced by other factors. The possibility that induction of BC1 RNA in tumors is due to a mutational change of cis-acting element(s) in or near the BC1 RNA gene could be ruled out since the

deregulation is also present in all cultured cell lines.

Repetitive sequences interspersed in the genome of eukaryotes were initially thought to be involved in the control of gene expression (Britten and Davidson, 1969; Davidson et al., 1973; Davidson and Posakony, 1982; Wharton et al., 1985; Glaichenhaus and Cuzin 1987). It has been shown that a large number of repetitive sequences containing transcripts are quantitatively regulated during normal mouse embryonic development and oncogenic transformation, and the abundance of these RNAs decreases during differentiation in vitro (Murphy et al., 1983, Scott et al., 1983, Vasseur et al., 1985). ID elements may act as positive regulators or enhancers of CAT and neo genes from a heterologous polymerase II promoter in mouse cells(McKinnon et al., 1986). As suggested by many others, ID sequences play a regulatory role in expression of differentiation-specific genes depending on the physiological state of the cell (active growth vs. growth arrest) (Lone et al., 1986; Herget et al., 1986; Glaichenhaus and Cuzin 1987). In this report, we first demonstrated that rodent BC1 RNA was deregulated in mouse tumor tissues. The possible role of BC1 RNA in the pathagensis of cancer tissues is an open question. The correlation between the induction of BC1 RNA and the multistep of neoplastic progression will be an interesting topic for further study.

Figure 15. Induction of Rodent BC1 RNA in Mouse Tumor Tissues.

a. Total RNA (10 μg per lane), except for both skin samples (5 μg per lane) was extracted from rat brain, different tumor tissues and their corresponding normal tissues, respectively. The RNA blot was prepared as described in Methods, and hybridized to probe HT005 which is complementary to the 3′ unique region of BC1 RNA. The RNA from rat brain was used as a positive control. A significant amount of BC1 RNA was detected in rat brain (RB), and mouse breast tumor (MBr-T), colon tumor (MC-T), and skin tumou (MS-T) but not in the respective normal tissues. (N: normal, T: tumor).

b. The same blot was rehybridized with β-actin cDNA probe. Similar intensities were observed in paired tissues (normal vs. tumor).

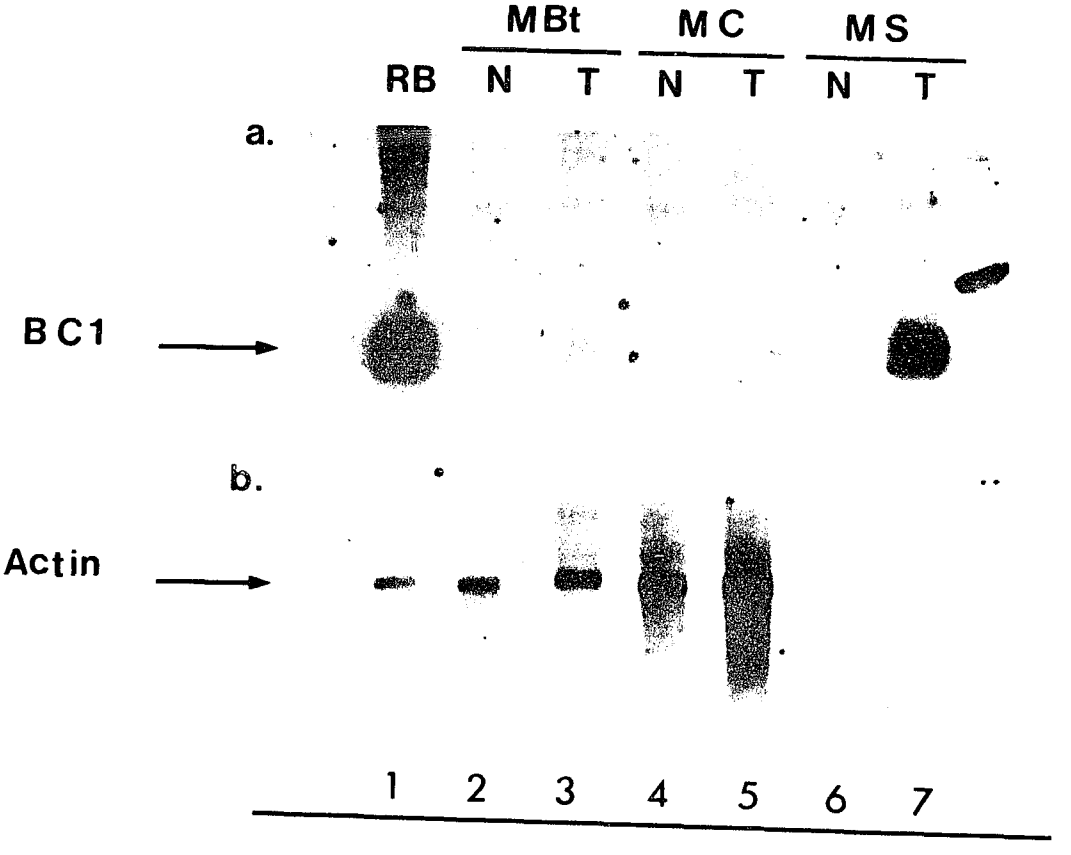
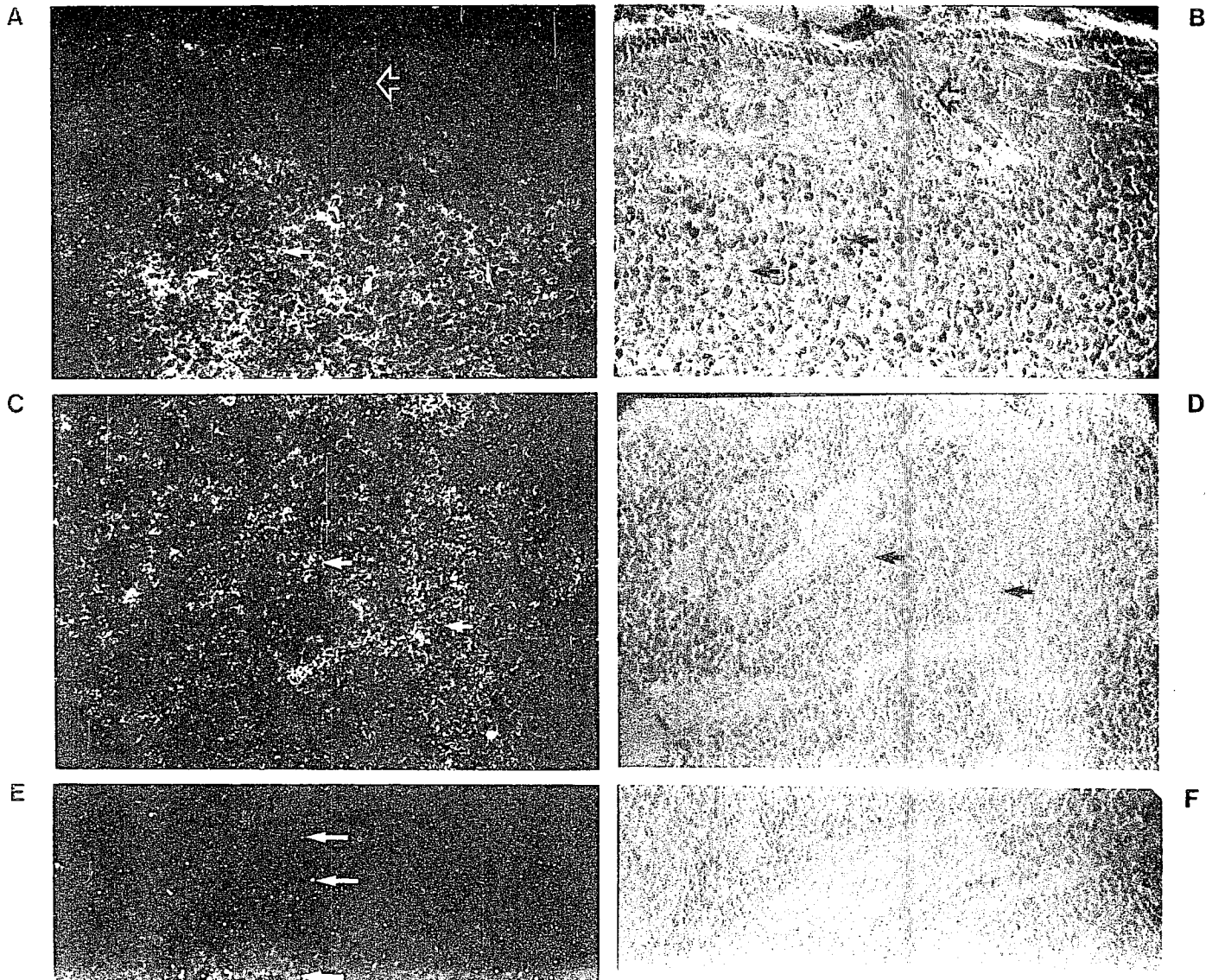

MBt
MC
MS
RB
N
T
N
T
N
T
a.
BC1
b.
Actin
1
2
3
4
5
6
7

Figure 16. Distribution of BC1 RNA in Mouse Skin and Colon Tumors.

Panel a and b: mouse skin tumor; panel c and d: mouse colon tumor; panel e and f: mouse colon tumor; A ^{35}S-labeled BC1 RNA probe ("antisense") was used in a and c. BC1 RNA "sense" control probe was used in e. BC1 RNA signal is observed in most of the tumor cells which indicted by solid arrows. None or little labeling is present in normal epidermis including rete ridges in a (open arrow).

In e, A "sense-strand" control probe was used to detected non-specific labeling. Silver grains are indicted by large solid arrows. Dark-field (a, c and e) and DIC (b, d and f) optics, Nikon Microphot-FX. Scale bar, 30 μm.


A
B
C
D
E
F

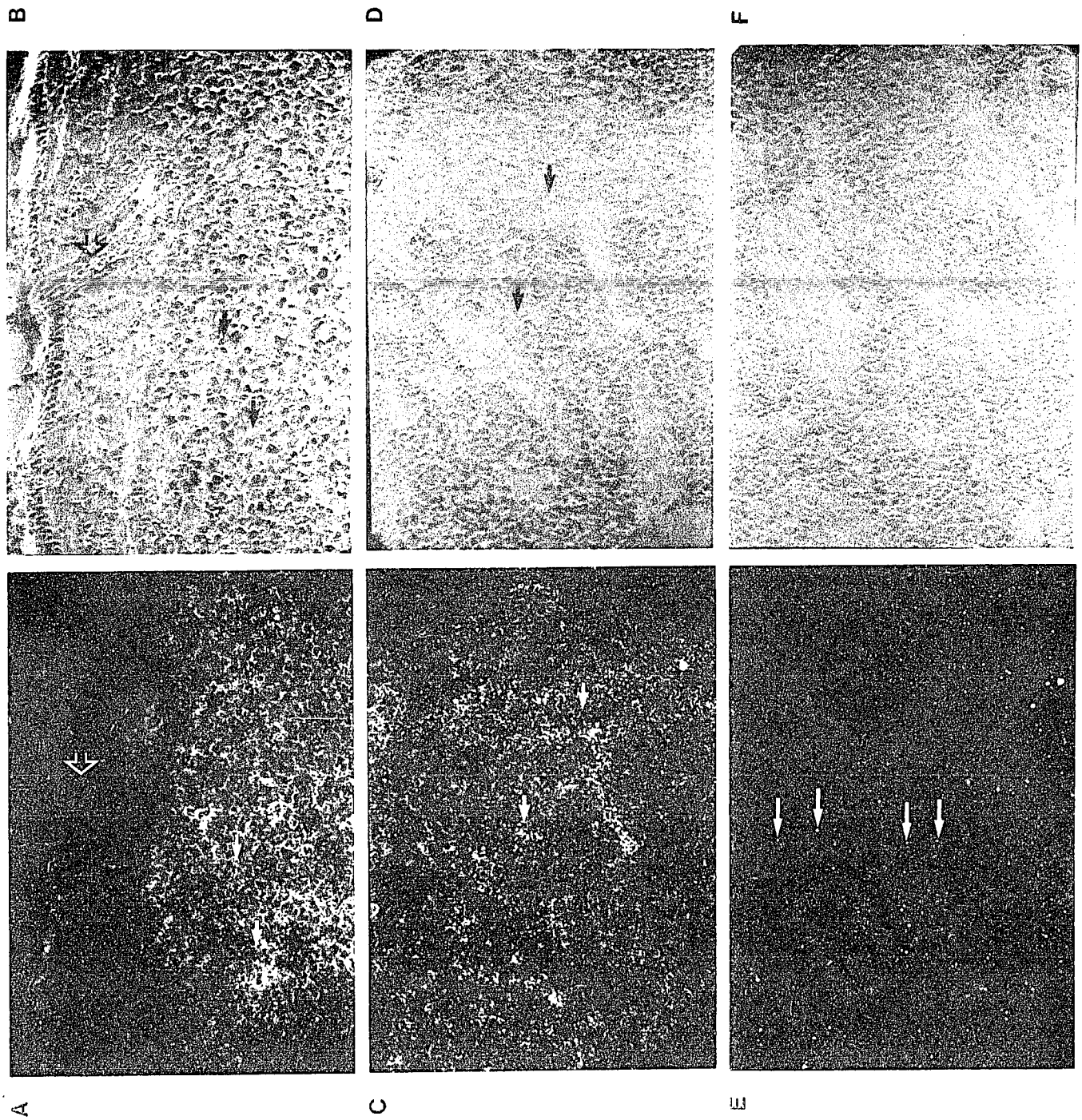
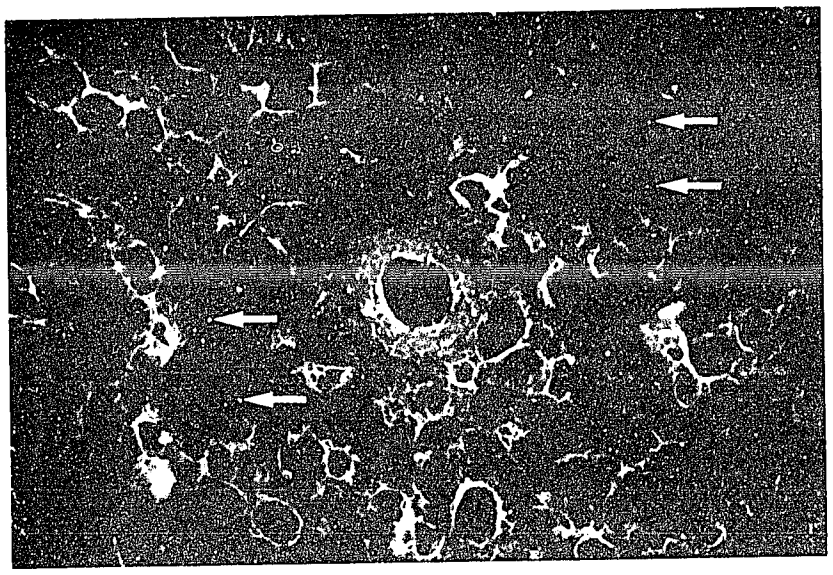
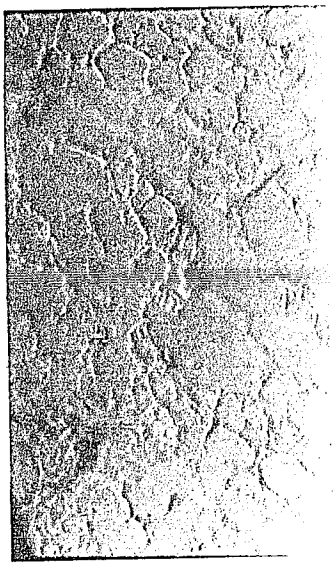

B
D
F
A
C
E

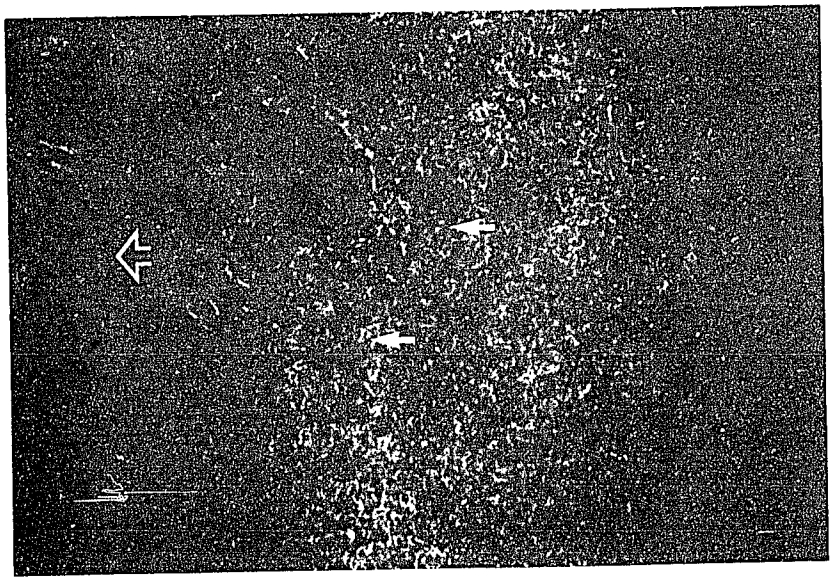
Figure 17. Distribution of BC1 RNA in Mouse Breast Tumor. Panel a and b: Normal structure of mouse breast; panel c and d: mouse breast tumor; White silver grains indicte BC1 RNA labrling. BC1 RNA signal is observed in one part of the tumor cells (solid arrows) while none or little labeling is present in other part (open arrow). In a, silver grains are indicated by large solid arrows.
Dark-field (a and c) and DIC (b and d) optics, Nikon microphot-FX. Scale bar, 30μm.

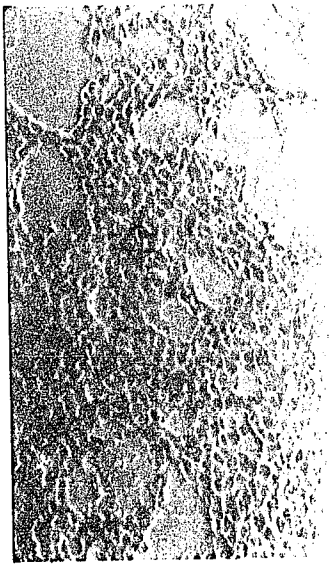
A

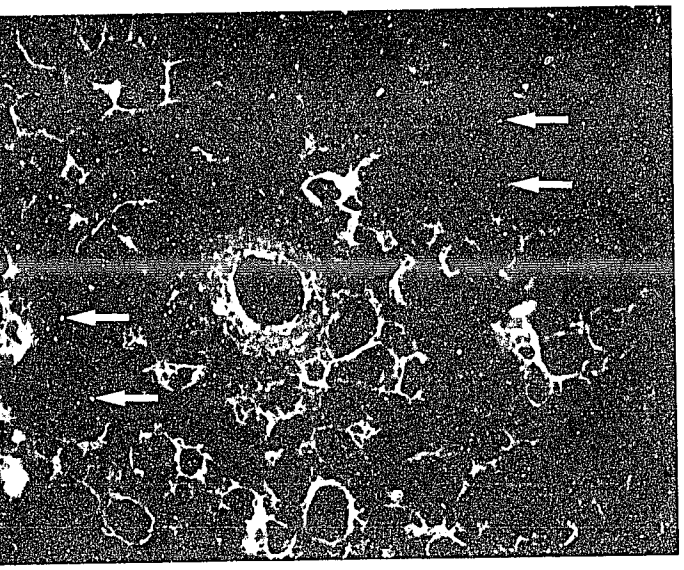


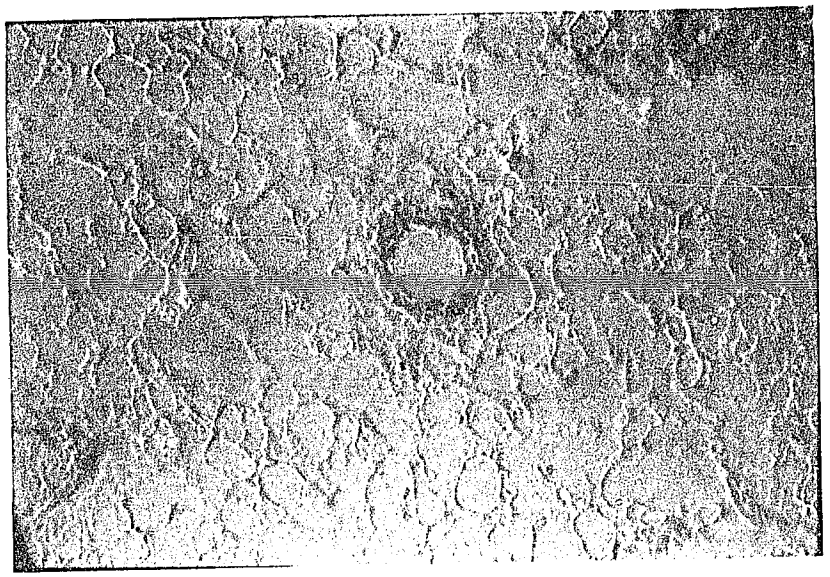


C

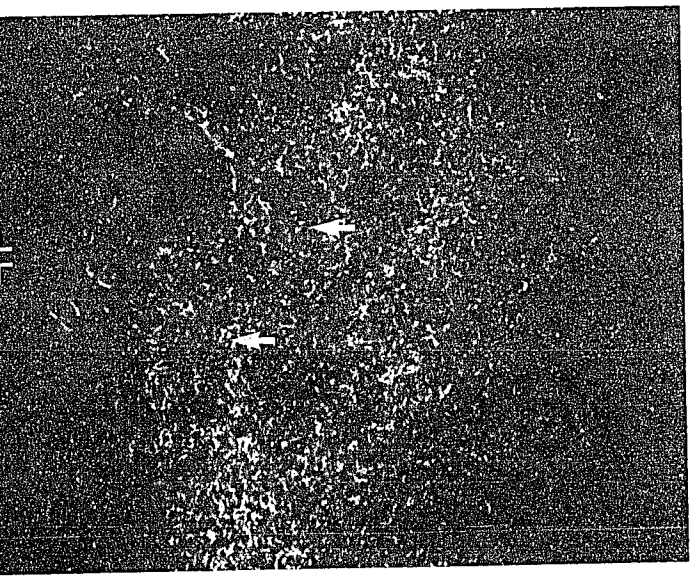


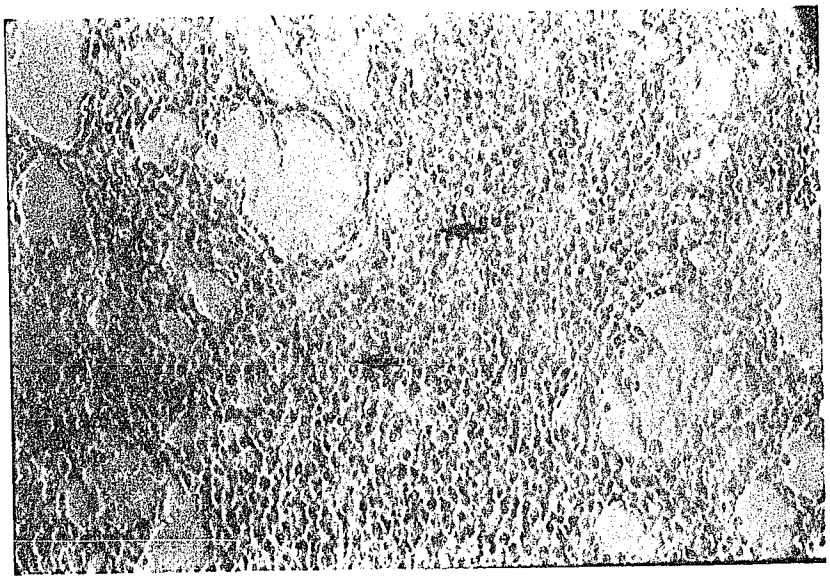






B





D

CHAPTER 7

EXPRESSION OF BC200 RNA IN HUMAN CARCINOMA TISSUES

ABSTRACT

Primate BC200 RNA is a 200-nucleotide-long, nontranslatable RNA that is almost exclusively expressed in nerve cells and developing germ cells in male gonad, and located in the dendrites and somata of a subset of neurons. It is the first identified RNA polymerase III transcribed neural specific RNA in primates and the first example of a transcriptionally and transpositionally active Alu element. It has been known that rodent BC1 RNA, analogous of BC200 RNA, as well as primate BC200 RNA are deregulated in immortalized cell lines. In addition, rodent BC1 RNA is induced in mouse tumor tissues. To investigate whether transcriptional regulation of BC200 RNA is also altered during tumorigenesis, tumor specimens from different human tissues were screened for the presence of BC200 RNA. Of the 80 tumor samples from 19 different organs analyzed in the study, significant amounts of BC200 RNA were detected in carcinomas of lung, breast, parotid, tongue, esophagus, ovarial and cervix, but not in their adjacent normal tissues. BC200 RNA is not present in colon, liver, kidney, bladder and other examined carcinoma tissues. These studies have demonstrated that BC200 RNA is deregulated during certain neoplastic conditions. Like BC1 RNA, the induction of BC200 RNA is tumor cell specific and might play a role in the pathogenesis of those cancer tissues under some circumstances.

INTRODUCTION

Human Alu sequences comprise an abundant family of interspersed repetitive elements, $5X10^5$-10^6 copies are present in the human genome (Jelinek et al., 1980; Jelinek and Schmid, 1982). In primates, the prevalent Alu consensus sequence is dimeric which consists of two imperfect monomeric repeats, left (Lm) and right monomeric (Rm) (Deininger et al., 1981). In contrast, the rodent Alu-equivalent, the B1 element, is monomeric (Krayev et al., 1980). These elements possess internal RNA polymerase III promoter sequences and represent retroposed pseudogenes ultimately derived from 7SL RNA, a component of the signal recognition particle (Ullu and Tschudi, 1984).

Primate BC200 RNA is a neural specific cytoplasmic small RNA and the first example of a neural specific, RNA polymerase III transcription product in primates (Watson and Sutcliffe, 1987). BC200 RNA can be subdivided into three structural domains. The 5′ domain shares 89% sequence similarity to the Alu Lm, followed by the central part of BC200 RNA which is an A-rich domain. The 3′ region is unique to BC200 RNA (Tiedge et al., 1993). It is the only known transcriptionally active Alu element and ultimately derived from 7SL RNA (Martignetti and Brosius, 1993a). The subcellular location of this small RNA, like BC1 RNA, the neural specific, polymerase III transcribed small RNA counterpart in rodents, is identified both in the

somatic and dendritic compartments of a subset of neurons (Tiedge et al., 1991, 1993). Furthermore, BC200 RNA is likely to be complexed with proteins to form a ribonucleoprotein particle (RNP)(Cheng et al., 1993). Therefore, it has been hypothesized that BC1 and BC200 RNAs, although of different evolutionary pedigree, may play analogous functional roles in local translation-related processes, in rodents and primates, respectively (Tiedge et al., 1991, 1993).

Previous studies have shown that the transcriptional activity of many class III genes was stimulated in rapidly growing cells such as tumors, serum-stimulated cells and several viral oncogene transformed cell lines (Singh et al., 1985; Edwards et al., 1985; Carey et al., 1986, 1988; Hoeffler et al., 1988; Tower and Sollner-Webb, 1988; Jang and Latchman, 1989; Panning and Smiley, 1993) and that the activities of general polymerase III transcription factors TFIIIB and TFIIIC are sensitive to growth condition and oncogenic transformation (Singh et al., 1985; White et al., 1989; Gaynor et al., 1985; Hoeffler et al., 1988; Tower et al., 1988). The expression levels of Alu elements is widely varying in different cell proliferation and differentiation states (Carey et al., 1986, 1988; White et al., 1989; Sakamoto et al., 1991). It has been reported that Alu elements and 7SL RNA modulate Hela cell growth (Sakamoto et al., 1991). In addition, transfection of mouse L-cells with certain Alu-rich genomic DNA clones can

elicit phenotypic change such as expression of the leukemia-related cell surface antigen in those cells (Chamberlain et al., 1986; Beitel et al., 1986).

It has been known that Both BC1 and BC200 RNAs are expressed in immortalized cell lines (Mckinnon et al., 1986, Watson and Sutcliffe, 1987). Our recent studies have shown that the rodent BC1 RNA is deregulated in viral oncogene and chemical carcinogen induced mouse tumor tissues (Chen et al., in preparation). In this report, tumor specimens from different human tissues were screened for the presence of BC200 RNA. Neural-specific BC200 RNA was deregulated in certain but not all examined carcinoma tissues. Our data suggest that the expression of BC200 RNA during carcinogenesis is not simply related to cell proliferation state, rather, it might be associated with specific protein factors which may be under- or over-expressed or deregulated in particular neoplastic tissue derived cells.

MATERIAL AND METHODS

Human tumor tissues and their adjacent normal tissues were provided by the Department of Pathology, Mt Sinai Hospital and Institute of Pathology, Medical School, Munster (Germany). The histological diagnosis was confirmed for each individual tissue block by staining with hematoxylin and eosin.

Preparation and analysis of RNA

Human tumor tissues and their adjacent normal tissues were removed and immediately frozen in liquid nitrogen. Total RNA was isolated by homogenization in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion. RNA (10μg) was fractionated on 1.8% agarose-formaldehyde gels, transferred onto GeneScreen membranes, immobilized by UV illumination and hybridized to oligonucleotide probes, BC207 and 7SL. BC207, complementary to the 3′ unique BC200 sequence 5′-CTTGTTGCTTTGA GCGAAGTTACGCTTATTTGGTAC-3′, was labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The same blots were then rehybridized to a 7SL probe which is complementary to the central non-Alu region of 7SL RNA 5′-CGAGGTCACCATATTGATGCCGAACTTAGTGGGTAC-3′. Hybridization was performed at 42°C in a buffer containing 1M NaCl, 0.5M Tris-HCl (pH 7.5), 5X Denhardt's reagent, 1% SDS and 0.1mg/ml yeast tRNA. The membranes were then washed three times at 55°C in 0.5X SSC and 0.1% SDS for 30 min.

In Situ hybridization

Fresh tissues from human tumor tissues and their adjacent normal tissues were quickly frozen using liquid nitrogen, embedded in Tissue-Tek OCT embedding medium, and then sectioned in a Bright Microtome Cryostat at 10 μm thickness. Sections were collected on gelatin and poly-L-lysine coated microscope slides.

The probe used to detect human BC200 RNA in the in situ

hybridization experiment corresponded to the sequence 5′-AAATAAGCGTAACTTCCCTCAAAGCAACAA-3′ in the 3′ unique region of BC200 RNA which was subcloned in the pBluescript KS(+) transcription vectors. ^{35}S- labelled RNA probes ("sense" and "antisense" strands) were transcribed from linearized templates using T3 or T7 RNA polymerase (BRL protocol).
In situ hybridization experiments were performed as previously described (Tiedge, 1991). This protocol used UV- light as cross linking agent to improve signal-to-background ratios.

RESULTS

Northern Blot Analysis

To determine whether the expression of BC200 RNA was deregulated during carcinogenesis, total RNA from different human cancer tissues and their adjacent normal tissues were analyzed for presence of BC200 RNA by Northern blot hybridization. RNA from human brain was used as positive control. In initial studies, a total of 35 tumor samples from 19 different organs have been screened (Table 3). Significant amount of BC200 RNA was detected in squamous cell carcinoma of lung, infiltrating duct carcinoma of breast, mucoepidermoid carcinoma of parotid and lung metastatic melanoma, but not in their adjacent normal tissues (Fig.18A). Low levels of BC200 RNA were detected in carcinomas of tongue, esophagus, stomach (tubular adenocarcinoma), ovarial, cervix and muscle sarcoma (Table 3). However, BC200

RNA was not present in carcinomas of thyroid, hypopharynx, stomach (adenocarcinoma), liver, colon, kidney, bladder, lung (adenocarcinoma) and non-hodgkin lymphoma (Fig 18B, Table 3). In contrast, 7SL RNA was slightly elevated in all examined tumor tissues (Fig. 18A, 18B). The data indicate that BC200 RNA is deregulated in certain types of human cancers.

Since BC200 RNA was consistently expressed in breast cancer from our initial screening (5 samples examined) (Table 3), we then focused on human breast carcinoma to examine whether BC200 RNA is expressed in all cases and whether there is a correlation between the patterns of the expression of this small RNA and histological grades of the tumors. Of the 34 breast samples analyzed by Northern blot hybridization, BC200 RNA was not detected in 8 cases (23%), whereas significant expression was observed in 21 cases, low levels in 5 cases (Table 4). There was no significant linkage between the expression of BC200 RNA and the histological grades of breast tumors (Table 4). In addition, 10 samples of lung squamous cell carcinoma were further analyzed by Northern blot hybridization. BC200 RNA was only present in 4 cases (25%) (data not shown).

In Situ Hybridization

The subcellular distribution of BC200 RNA in tumor tissues was examined by in situ hybridization. The ^{35}S-labeled antisense

RNA probe was generated complementary to part of the unique 3′ domain of BC200 RNA. The sense strand probe, corresponding to this portion of BC200 RNA, was used as a negative control (see methods for detail). In general, the expression pattern of BC200 RNA was similar from case to case which was restricted to the tumor cells. The majority of malignant cells expressed BC200 RNA. None or little labeling has been observed in the intratumor stroma connective tissue that separated malignant lesions. Representative in situ hybridizations are shown in figure 19. In breast carcinoma (Fig 19A and B), BC200 RNA signal was mainly located in the cords and clumps of the tumor cells. The squamous cell carcinoma of lung showed a similar staining pattern (Fig. 19C and D). For invasive squamous cell carcinoma of tongue, labeling was concentrated on sheets of the tumor cells (Fig. 19E and F). The " sense strand" control probe failed to produce specific labeling in any of the examined tissues (data not shown).

DISCUSSION

In this paper, we demonstrate that BC200 RNA is deregulated in certain human tumor tissues but not in the adjacent normal tissues. It is noteworthy that, in breast cancer which we studied extensively, this small RNA is not present on all (23%) and only a fraction of the samples studied (77%). BC200 RNA is detected exclusively in malignant lesions while none or little labeling has been observed in intratumor stroma

cells. Therefore the tumor cells are shown to be the predominant if not only sites for expression of the BC200 RNA. In contrast, 7SL RNA was slightly elevated in all examined tumor tissues.

BC200 RNA is the first identified neural specific RNA polymerase III transcribed product in primates (Watson and Sutcliffe, 1987). Like other tissue-specific genes, DNA elements (positively and negatively acting element) as well as their cognate transcriptional factors mediate neural specific regulation (Struhl 1991; Mandel and Mckinnon 1993). In particular, the suppressor-like elements appears to be important for the majority of neural-specific genes that have been studied to date. Therefore the presence of specific protein activator(s) or absence of silencer-binding protein in neurons, in addition of general pol III transcription factors (TFIIIB, TFIIIC), might contribute to the neural-specific transcription of the BC200 RNA gene. It is possible that under some circumstances of carcinogenesis, those factors could be directly or indirectly under- or over-expressed or deregulated in cancer tissues resulting in activation or deinhibition of the BC200 RNA gene promoter. Alternatively, the stability of this small RNA might be enhanced by certain factors. The possibility of an increase in BC200 RNA transcription due to a mutational change of cis-acting element(s) of BC200 RNA gene can be ruled out since the

induction is present in all cultured cell lines.

7SL RNA is also a RNA pol III transcribed cytoplasmic small RNA which is constitutively expressed in all tissues. The 7SL domain homologous to the Alu sequences is responsible for the binding of protein components to form the signal recognition particle (SRP) that is involved in the elongation arresting activity (Walter et al., 1982, 1984). It has been known that RNA polymerase III transcription factors are subject to growth-related controls. Alterations in TFIIIC, both quantitative and qualitative, accompany alterations in growth state or transformation of cells by viral genes and oncogenes (Yoshinaga et al., 1986; Hoeffler et al., 1988; Carey et al., 1988; Jang and Latchman 1992). Infection with herpes simplex virus (HSV), adenovirus E1A as well as in SV40-transformed cells result in an increased activity and amount of TFIIIC (Yoshinaga et al., 1986; Hoeffler et al., 1988; Carey et al., 1988; Jang and Latchman 1992). Unlike BC200 RNA, 7SL RNA is slightly elevated in all samples studied which could be attributed to increased activity of TFIIIC and TFIIIB, general pol III transcription factors.

Many studies have been carried out to show that the levels of pol III transcribed small Alu RNAs were altered by different stimuli. DNA virus HSV immediate-early protein ICP27 (Jang and Latchman, 1989, 1992) and adenovirus E1B gene products (

Panning and Smiley, 1993) stimulate pol III transcription of human Alu elements. It has been hypothesized that the Alu element complexed with protein factors, related to that of the signal recognition particle (SRP) containing 7SL RNA, may be involved in elongation arrest of viral mRNA during infection (Jurka 1989; Jurka et al., 1991). It also has been reported that transfected 7SL RNA and Alu sequences suppress Hela cell growth (Sakamoto et al., 1991). Our data show that the BC200 RNA gene is selectively expressed in certain neoplastic diseases and restricted to tumor cells. These findings imply that the deregulation is not solely a nonspecific reaction and that it might contribute to tumor suppression under some circumstances, although this hypothesis remains to be tested experimentally.

Our recent studies show that rodent BC1 RNA, analogous to primate BC200 RNA, is deregulated in mouse tumor tissues (Chen et al., in preparation). Although the staining pattern varied greatly from just a few scattered, positive cells to extensive staining of the most malignant lesion, it is often heterogeneous within the same sample. Like BC200 RNA, BC1 RNA signal is confined to tumor cells not to cells within the stroma. The data suggest that both BC1 and BC200 RNAs might play the same role in the pathogenesis of certain cancer tissues. The possible correlation between the expression of BC200 RNA and other oncogenes (Ki-ras, c-myc, int2, c-erbB2

and c-raf) as well as the tumor progression will be investigated in the future.

Figure 18. Induction of Primate BC200 RNA in Human Tumor Tissues.

Total RNA (10 μg per lane) from human brain, different human tumor tissues and their adjacent normal tissues was prepared for Northern blot analysis. RNA blots were hybridized with probe BC207 (upper panel of A and B) which is complementary to the 3′ unique region of BC200 RNA. A positive signal is observed in human brain (HB), squamous cell carcinoma of lung (HLu-T), carcinoma of breast (HBr-T), mucoepidermoid carcinoma of parotid (HP-T), and lung metastatic melanoma (HM-T). BC200 RNA was not detected in carcinomas of liver (HL-T), bladder (HBd-T), kidney (HK-T), colon (HC-T) and all their adjacent normal tissues. (N: normal, T: tumor). The same blot was rehybridized with a probe complementary to 7SL (the central non-Alu region) (bottom panel of A and B). 7SL RNA is present in all tissues and the expression level is elevated in all carcinoma tissues comparing with their adjacent normal tissues.

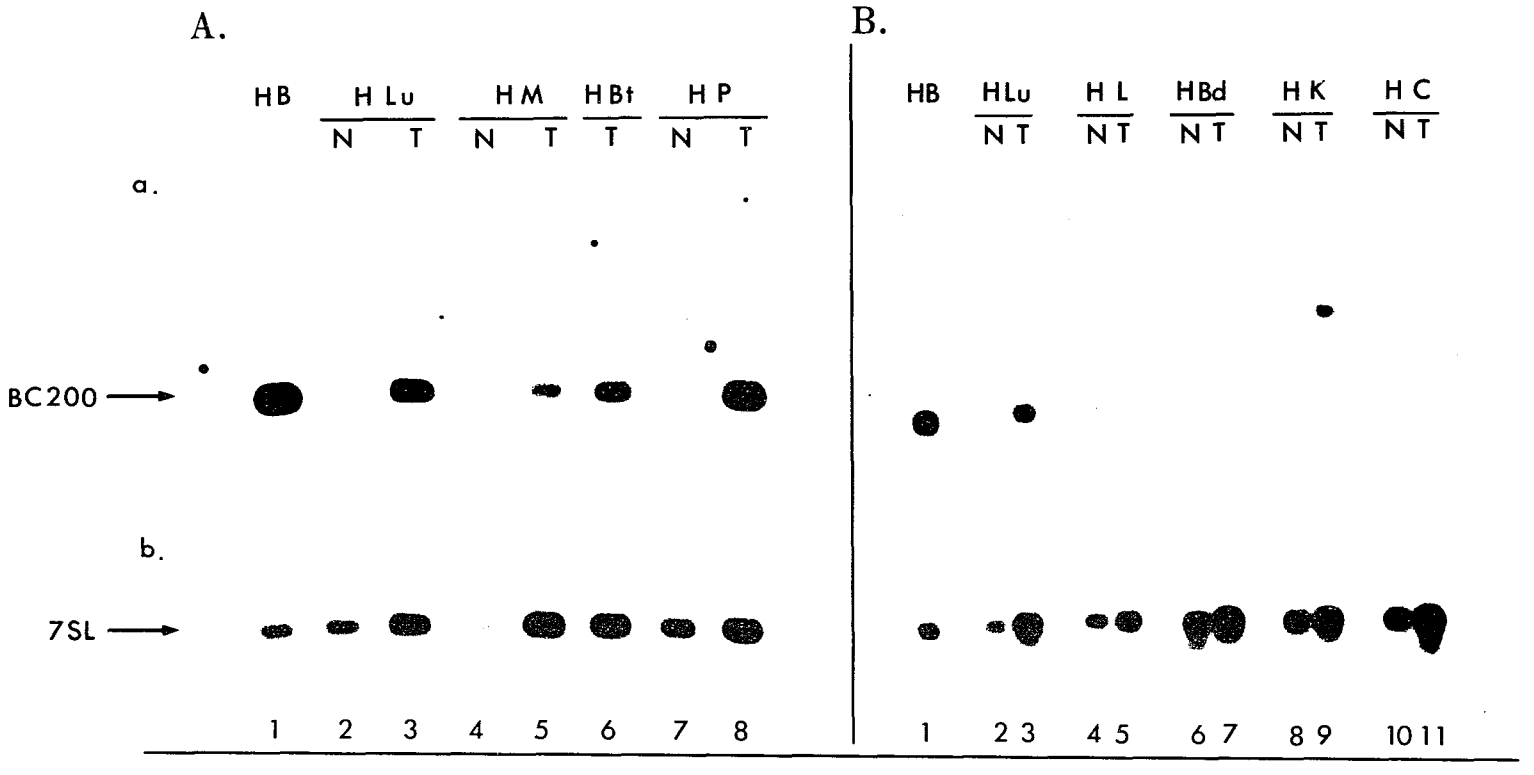
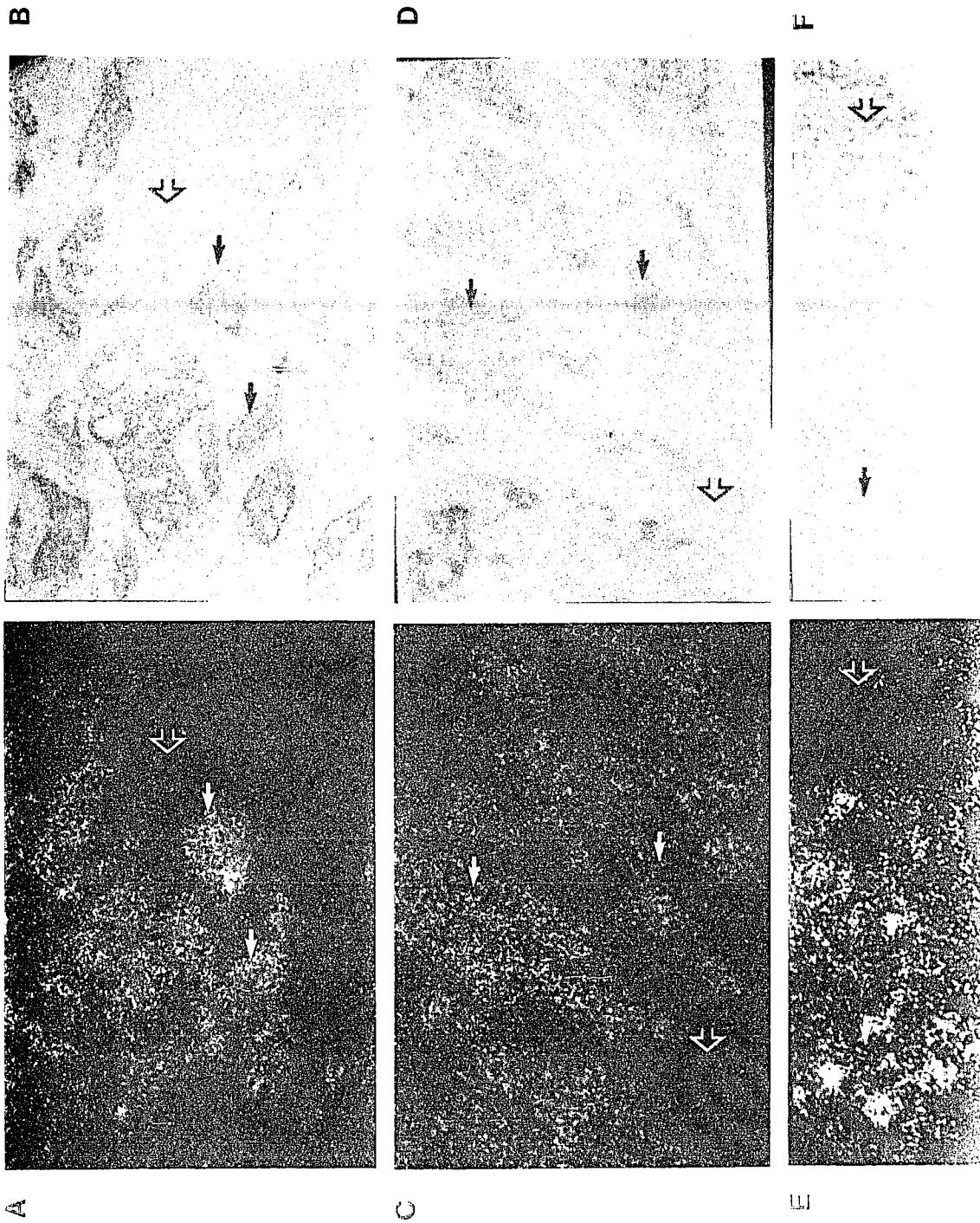

A.
B.
HB
H Lu
N T
H M
N T
HBt
T
H P
N T
HB
HLu
N T
H L
N T
HBd
N T
H K
N T
H C
N T
a.
BC200
b.
7SL
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8 9 10 11

Figure 19. Distribution of BC200 RNA in Human Tumor Tissues. Panel a abd b: human breast carcinoma; panel c and d: human squamous cell of lung carcinoma; panel e and f: human squamous cell carcinoma of tongue; White silver grains indicte BC200 RNA labeling. Sections were stained with cresyl violet. BC200 RNA signal is restricted to the malignant lesions (solid arrows). No or little labeling is observed in the intratumor stroma cells (open arrows).

Dark-field (a,c and e) and bright-field (b,d and f) were taken with Nicon Microphot-FX. Scale bar; 30 μm for c, d, e and f. 60 μm for a and b.


B
D
F
A
C
E

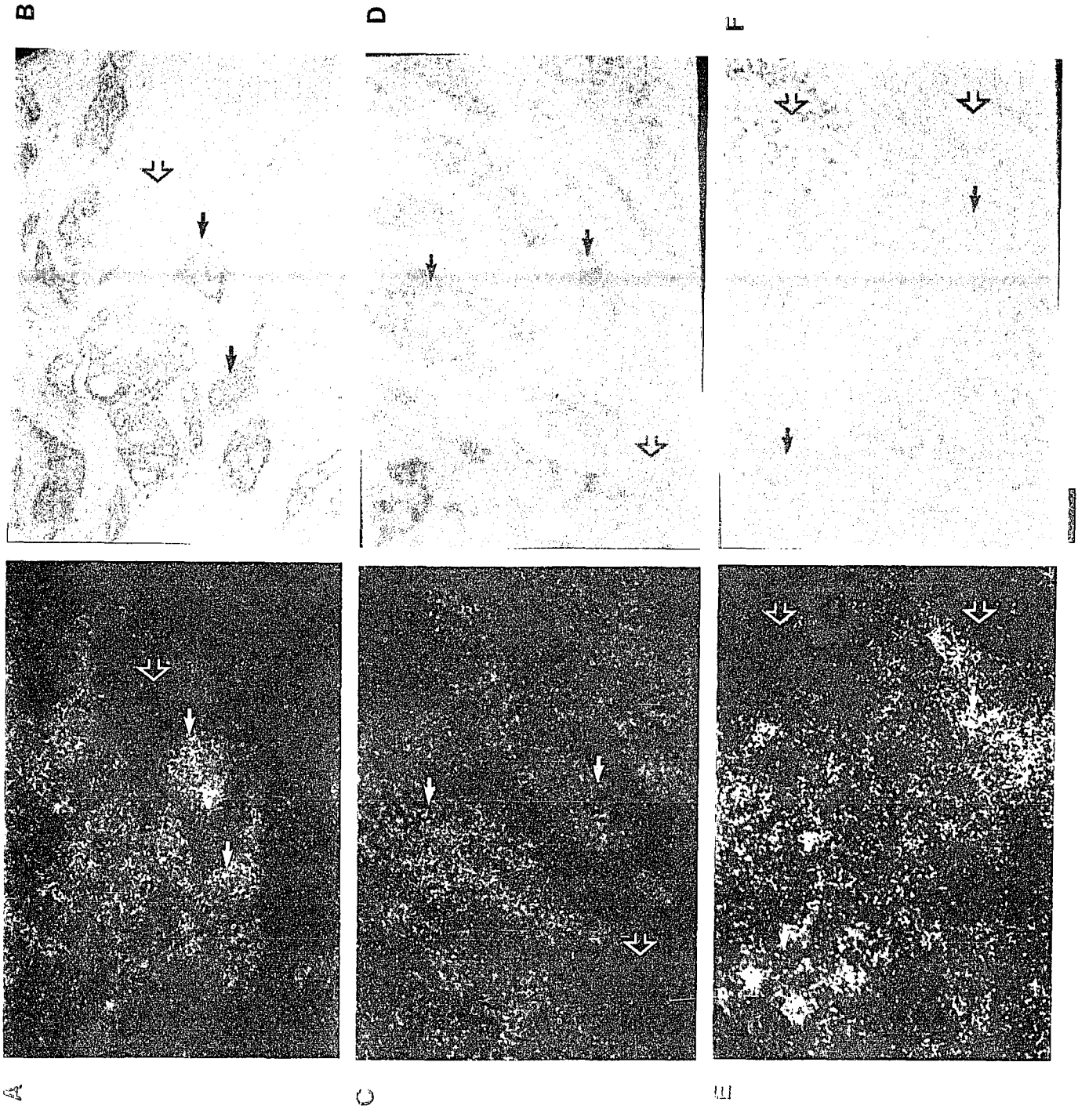

B
D
F
A
C
E

TABLE 3. EXPRESSION OF BC200 RNA IN DIFFERENT HUMAN TUMOR TISSUES AND DIFFERENT CELL TYPES

TISSUE SOURCE	NO. OF CASES	CELL TYPE	BC200EXP.
HYPOPHARYNX	1	EPITHELIAL TUMOR	-
TONGUE	1	SQUAMOUS CELL CARCINOMA	+
ESOPHAGUS	1	EPITHELIAL CARCINOMA	+
STOMACH	2	ADENOCARCINOMA	-
	1	TUBULAR ADENOCARCINOMA	+
LIVER	4		-
RECTOSIGMOID COLON	2	ADENOCARCINOMA	-
HEMICOLECTOMY	1	ADENOCARCINOMA	-
COLON	5	ADENOCARCINOMA	-
KIDNEY&ADRENAL GLAND	1	CLEAR CELL	-
BLADDER	1	TRANSITIONAL	-
BREAST	5	ADENOCARCINOMA	+++
LUNG	1	SQUAMOUS	+++
	2	ADENOCARCINOMA	-
PAROTID GLAND	1	MUCOEPIDERMOID	+++
THYROID GLAND	1		-
MUSCLE&SKIN	1	SARCOMA	+
LUNG METASTASIS (MELANOMA)	1	MELANOMA	++

OVARIAL	1	PAPILLARY CARCINOMA	+
CERVIX	1	ENDOTHIAL ADENOCARCINOMA	+
NON-HODGKIN LYMPH.	1	T CELL TYPE	-

TABLE 4. EXPRESSION OF BC200 RNA IN HUMAN BREAST CARCINOMA

#	BC200	PATH
1241	-	poorly differentiated infiltrating duct carcinoma
810	-	poorly differentiated infiltrating duct carcinoma
1007	-	moderately to poorly differentiated infiltrating carcinoma
76	-	No
162	-	infiltrating carcinoma predominantly lobular with some ductal features
692	-	No
719	-	No
337	-	poorly differentiated infiltrating and intraductal duct carcinoma
534	+	infiltrating ductal carcinoma with axillary nodes metastasis
793	+	moderately differentiated infiltrating duct carcinoma
1134	+	moderately and poorly differentiated duct carcinoma
223	+	No
968	+	moderately differentiated infiltrating and intraductal carcinoma
1050	++	No
16	++	No
488	++	No
1166	++	moderately differentiated infiltrating duct carcinoma
105	++	mass right axilla
702	++	No
1185	++	left axilla node
636	++	No
1104	++	No
197	++	No

521	++	large colloid carcinoma with direct invasion of skin
157	++	No
1092	++	No
669	++	No
1069	++	No
1010	++	moderately to poorly differentiated infiltrating duct carcinoma
54	++	moderately differentiated intraductal and infiltrating duct carcinoma
661	+++	invasive ductal carcinoma
374	+++	ductal carcinoma with bone metastasis
608	+++	No
834	+++	No

CHAPTER 8

DISCUSSION

As a step toward understanding the mechanisms regulating neuron-specific gene expression, we chose first to study the regulatory sequences of the neuron-specific BC1 gene as a main emphasis of this work. Rodent BC1 RNA represents the first example of a neural-specific RNA polymerase III transcription product and therefore provides a model for studying both RNA polymerase III and neural-specific transcription regulation.

Introduction of the 1.4 Kb rat BC1 gene, containing RNA polymerase II (two octamer binding sequences, PSE and TATA box) as well as polymerase III (A and B boxes) promoter elements, into the mouse germline has resulted in efficient levels of expression in all three lines of transgenic mice and, with the exception of testes, it was restricted to the nervous system. It was not found in liver, lung, spleen, heart, kidney and other non-neuronal tissues. The expression pattern of the transgene and endogenous BC1 gene was shown to be parallel even in their subcellular localization: dendrites and somata of neurons. These results suggest that cis-acting regulatory elements sufficient to targeting of neuron-specific expression are located within 1.4 Kb of the

rat BC1 gene. In addition, a similar pattern of expression in all three transgenic lines indicates that transgene expression appears to be independent of chromosomal integration sites (see chapter 3). To extend these studies, additional transgenic mouse lines with a deletion of 5′ distal end of 1.4 Kb gene (BC1-5′) have been characterized. Deletion of 357 bp including two octamer motifs in the proximal 5′ flanking sequence (from -429 to -72) has no effect upon the tissue specificity but the average level of expression per transgene copy is about 5% when compared to endogenous mouse BC1 RNA and about 5 times less than the 1.4 Kb rat BC1 gene. Furthermore, the integration site-independence disappeared. Therefore the sequence (from -429 to -72) of rat BC1 RNA gene is not essential for the neural specific expression of BC1 RNA. It might contain a neural specific or general enhancer. In addition, integration site-independent expression of BC1 transgene requires the presence of 357 bp fragment of the 5′ flanking sequence (see chapter 5). It is not clear which elements within the remaining 1 kb construct containing 72 bp of 5′-flanking and 870 bp of 3′-flanking sequences are responsible for BC1 RNA gene neural-specific expression or whether they are internal or external to the gene (or both). To answer this question, further deletions and alteration of this construct will be employed to localize those sequences. Taken together, the appropriate tissue-specific expression of the 1 kb fragment of BC1 gene in transgenic mice provides the

potential opportunity of identifying sequences which participate in neuronal specific regulation.

In the second part of these studies, I described that both BC1 and BC200 RNAs, neural-specific pol III transcripts, can be deregulated during certain carcinogenesis. Both RNAs are detected exclusively in malignant lesions where no or little labeling has been observed in their adjacent normal tissues and intratumor stroma cells. Therefore the tumor cells are shown to be the origin for transcription of BC1 and BC200 RNAs. Since the deregulation is only observed in a fraction of the samples studied this indicated that the induction of BC1 and BC200 RNAs during carcinogenesis is not simply related to cell proliferation state. Deregulation might be associated with specific activator(s) or inhibitor(s) which may be under- or over-expressed or deregulated in particular neoplastic tissues, the promoter of both BC1 and BC200 genes could be activated under those condition. Alternatively, the stability of RNAs might be enhancered by some factors (see chapter 6 and 7). The possibility of an increase in both RNAs transcription due to a mutational change of cis-acting element(s) can be ruled out since the induction is observed in all cultured cell lines.

It has long been speculated that the repetitive elements (SINEs), like ID and Alu sequences in rodent and primate

respectively, are involved in modulation of cell growth and play a regulatory role in differentiation-specific genes expression. On the other side, the expression level of these repetitive elements are regulated in different cell proliferation and differentiation states. Both BC1 and BC200 RNAs are ID and Alu repetitive sequences containing small RNAs. Although of different evolutionary pedigree, they may play analogous functional roles, in rodents and primates, respectively (see chapter 1, 6 and 7). Our findings imply that the induction of BC1 and BC200 RNAs are not solely a nonspecific reaction, as discussed in chapter 6 and 7, it may play a role in the pathagensis of cancer tissues under some circumstances.

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