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REGIONAL LOCALIZATION AND DEVELOPMENTAL EXPRESSION OF THE *BCR* GENE IN RODENT BRAIN

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Abstract

The *BCR* gene is implicated in the development of *Ph*-positive leukemia through its fusion with the nonreceptor tyrosine kinase gene *ABL*. The normal 160 kDa Bcr protein has several functional domains, and recently one specific role for Bcr was established in the regulation of respiratory burst activity in white blood cells. *Bcr* expression levels are relatively constant throughout mouse development until adulthood in brain and in hematopoietic tissues, a pattern that is distinctly different from that of the functionally related *n-chimerin* gene. In the present study, RNA in situ hybridization was used to explore the normal cellular function of Bcr in rodent brain and hematopoietic organs. The data pinpoint the high *bcr* expression in the brain to the hippocampal pyramidal cell layer and the dentate gyrus, and to the piriform cortex and the olfactory nuclei, reflecting a potentially interesting function for Bcr in these highly specialized brain regions.

Keywords

bcr; Brain; Expression; Mouse

INTRODUCTION

The human *BCR* locus was first identified in a *b*reak-point *c*luster *r*egion on chromosome 22 in Philadelphia (*Ph*) positive leukemia (Groffen et al., 1985; Heisterkamp et al., 1983): through a t(9;22)(q34;ql 1) translocation, the *BCR* gene becomes fused with the *ABL* gene, a nonreceptor tyrosine kinase normally located on chromosome 9 (Heisterkamp and Groffen, 1991; Rowley, 1990). The leukemogenic role of *BCR/ABL* has been demonstrated experimentally in transgenic mice carrying *BCR/ABL* fusion-transgenes and by retroviral transduction experiments in which mice receive *BCR/ABL*-positive bone marrow transplants (Daley et al., 1990; Elefanty et al., 1990; Heisterkamp et al., 1990; Voncken et al., 1992).

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The *BCR* gene encodes a 160 kDa cytoplasmic protein with several functional domains and is a protein likely to participate in signal transduction. Its *N*-terminal domain encoded by exon 1 has a serine/threonine kinase activity, can oligomerize, and is capable of binding other protein factors, including p145*Abl* (McWhirter et al., 1993; Sawyers et al., 1991). The central part of Bcr has homology to the G-nucleotide exchange factor (GEF) *Dbl,* and the carboxy terminal part is homologous to the n-chimerin protein, which is selectively expressed in neuronal tissue (Diekman et al., 1991; Ron et al., 1991). As is the case for n-chimerin, this domain of Bcr has GTP-ase activating protein (GAP) activity toward a subfamily of p21*Ras*-related small GTP-binding proteins, p21*Rho/Rac* (Diekman et al., 1991). Recently, we have established at least one function for Bcr as a regulator of p21*Rac*-mediated superoxide production by the NADPH-oxidase in leukocytes (Voncken et al., 1995).

In the mouse, *bcr* expression is detectable as early as the zygote stage, continues throughout embryogenesis (unpublished observations), and is found in most adult tissues examined (Heisterkamp et al., 1993). In man, *BCR* has also been shown to be ubiquitously expressed. Notwithstanding its role in normal and leukemic blood cells (Campbell and Arlinghaus, 1991; Collins et al., 1987; Voncken et al., 1995), the Bcr protein is found in high levels in the brain. This observation has intrigued us as it suggests a possible different function for p160*Bcr*. This notion is corroborated by the seemingly highly significant function of G-proteins in the brain (Hopkin, 1994). In an attempt to further delineate a putative cellular function of Bcr in the brain, we have studied the regional expression of *bcr* in the rodent brain by means of RNA in situ hybridization. We also report data on the expression kinetics of *bcr* during mouse development in hematopoietic organs and in the brain.

MATERIALS AND METHODS

Tissue preparation

Paraffin embedded adult mouse brain sections were obtained from Novagen Inc. (Madison, WI). Adult Sprague–Dawley rats were decapitated rapidly and the brains were removed onto dry ice and stored at −80°C. Coronal sections (20 μm) were mounted on gelatine-coated slides and stored at −80°C.

Probes

A 400-bp fragment of mouse *bcr* was amplified by RT/PCR with the primers ALLE (Kawasaki et al., 1988) and BEX3 (5' AACCCACTTTCTCATCTCCAG 3') as previously described.(Heisterkamp et al., 1990). The RT/PCR product was gel purified and digested with EcoRI and Clal yielding a 251 -bp fragment that was subcloned directionally into pSK (Stratagene). The identity of the cloned fragment was confirmed by sequencing both strands using the Sequenase 2.0 system (USB). To obtain antisense and sense RNA probes, the construct was linearized with BamHI (antisense) or Xhol (sense) and transcribed in vitro with α-^{35}SUTP using T7 and T3 RNA polymerase, respectively, essentially as described by the manufacturer (Novagen Inc., Madison WI). A second *bcr* antisense RNA probe was produced by digesting a 1 8-kb HindIII/EcoRI genomic fragment (Zhu et al., 1990) cloned in pSK with Narl and transcription with T7 RNA polymerase. Probes were labeled to high specific activity, typically 2.0×10^8 cpm/μg. The oligonucleotide probe BEX2 (5' GAGGAAGAAGGTGAATCATCG 3') was labeled on the 3' end with ^{35}SdATP using terminal deoxy-nucleotidyl transferase (Bethesda Research Lab.). An oligonucleotide probe for an unrelated gene, CRH, was similarly generated and labeled as specificity control (Baram and Schultz, 1991; Yi et al., 1993).

In situ hybridization procedures

In situ hybridization on paraffin-embedded mouse tissues was performed using the SureSiteII System from Novagen. Briefly, paraffin was removed by three washes in xylene and rehydrated through a graded ethanol series. Tissue pretreatment consisted of ribosome disruption in 0.2 N HC1 for 5 min, deproteinization in 1 μg/ml proteinase K for 5 min, followed by acetylation in 0.1 M triethanolamine-HCl, pH 8.0, containing 0.25% (v/v) acetic anhydride for 5 min. Prehybridization, hybridization with antisense and sense probes on parallel sections, and subsequent washings were performed according to the manufacturers instructions. Slides were apposed to XAR2 films for 1–2 d, then dipped into Kodak NTB-2 emulsion, exposed in a sealed container for 2−3 weeks at +4°C, and finally developed and fixed using Kodak developer D-19 and Kodak fixer, respectively. Slides were stained in 5% aqueous Giemsa, examined and photographed with bright and darkfield optics using an Olympus BH-2 microscope equipped with an Olympus C-35AD-2 camera. In situ hybridization on rat brain sections using the oligonucleotide probes described above was performed essentially as previously described (Baram and Schultz, 1991, 1992; Yi et al., 1993). After prehybridization for 1 h and hybridization for 20 h at 40°C in a humidity chamber, serial washes were performed (4 washes at 2×SSC for 15 min at 40°C, followed by one wash in 1×SSC and one in 1×SSC at room temperature), followed by dehydration through ethanol-0.3M NH_4Ac and apposition to X-ray film (Hyperfilm B-max, Amersham) for 24–48 h.

Northern blot analysis

Tissues from C57B1/6 × B6CBA F1 mice were minced in a polytron, and total RNA was extracted using the acid guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). At embryonic day E15, E17, and E20 the head region was separated from the remainder of the embryo. At parturition (P0) and postnatal day 1, 5, 10, 15, 20, 25, 30, and 35, the entire brain including cerebellum was dissected. To compare the developmental expression of *bcr* in the brain with *bcr* expression in hematopoietic tissue, RNA was prepared from the liver at E15, E17, E20, at parturition, and from the spleen at postnatal day 1, 5, 10, 15, 20, 25, 30, and 35. Samples (10 μg) were run on formaldehyde/formamide gels, blotted as described (Sambrook et al., 1989), and subsequently hybridized to the 1.8-kb HindIII/EcoRI probe containing coding sequences from mouse *bcr* exon 1. Filters were washed at low stringency (2.5×SSC, 65°C for 20 min). At this stringency the probe also cross-hybridizes to the 28S and 18S ribosomal bands, making it possible to check for equal loading of the lanes. Densitometric scanning was performed using an in-house manufactured densitometric device.

RESULTS

In situ hybridization patterns

Coronal sections of adult mouse brain revealed specific hybridization mainly localized to cells of neuronal morphology when hybridized with either of the two (nonoverlapping) antisense bcr probes described above. The sense probe did not produce any specific signal in any of the experiments performed. High concentration of *bcr* mRNA was present in hippocampus, and to a somewhat lower degree in the cerebral cortex. In hippocampus and dentate gyrus, region CA1–CA3, the pyramidal cell and granular cell layers were strongly labeled (Figs. 1A and 1B). In the cortex, neurons in layers II–VI were moderately labeled (not shown), whereas a stronger labeling was noted in the piriform cortex (Fig. 1C). Some thalamic nuclei showed a weak hybridization (not shown). Neurons in amygdala and hypothalamus were not labeled above background hybridization. The hybridization pattern in the cerebellum was not investigated. In the rat, anterior olfactory nuclei displayed a specific hybridization pattern (Fig. 1C). In situ hybridization with labeled oligo-nucleotides

further revealed similar expression patterns to that of the mouse: dentate gyrus granular cells, the hippocampal pyramidal layer (CA1–CA3 region), and the piriform cortex clearly displayed a specific hybridization pattern, diminishing in intensity in the order indicated above (Fig. 2).

Gene expression throughout development

Northern blot analysis of *bcr* expression in the mouse brain showed a transcript of approximately 7 kb that was present at E15 and remained constant throughout the embryonal period of development, as well as through postnatal days 1–35 (Fig. 3). Densitometric scanning revealed no clear changes in *her* expression level over time, when corrected for unequal loading on the basis of background hybridization to 18 and 28S ribosomal RNA. The role of Bcr in blood cells has recently been validated (Voncken et al., 1995). Northern blot analysis of liver and spleen samples, organs that have a distinct hematopoietic function during embryogenesis and for some period after birth, showed a lower level of *bcr* expression throughout the same developmental stages, but, similar to *bcr* expression in the brain, the level remained constant throughout pre- and postnatal development (data not shown).

DISCUSSION

The Bcr protein is a multidomain protein and is involved in *Ph*-positivc leukemogenesis through its fusion to the nonreceptor tyrosine kinase Abl (Heisterkamp and Groffen, 1991; Van Etten, 1993). Although we have recently established a function for Bcr in neutrophils, the reason why *bcr* is highly expressed in the brain remains elusive. We have used in situ hybridization to study *bcr* expression patterns in the rodent brain. Our data show that *bcr* is highly expressed in the hippocampus and dentate gyrus, and to a lower level in the piriform cortex and anterior olfactory nuclei.

Granule cells of the dentate gyrus are primarily generated postnatally. In rodents, granule cells proliferate and differentiate almost exclusively in the first 2 postnatal weeks. In the mouse and the rat, a small percentage of these cells is constantly generated in the adult (Gage, 1994; Gould et al., 1994; and references therein). The hippocampus and olfactory bulb are sites of postnatal neurogenesis and intense synaptic plasticity. Small GTP-binding proteins as well as their regulatory molecules are frequently implicated in processes that control cellular growth in tissues, including the brain. The constitutive activation of p21*RAS* in *neurofibromatosis* by deactivation of neurofibromin (Basu et al., 1992; Bollag and McCormick, 1992; DeClue et al., 1992), a GTP-ase activating protein (GAP) for RAS, is a good example. The *BCR* gene itself has been implicated in human leukemia, and the *BCR*-related *ABR* gene appears to be deleted in particular cancers (Isomura et al., 1994; Morris et al., 1995; Saxena et al., 1992).

Although we cannot entirely exclude an involvement of Bcr in neuronal proliferation, the developmental profile of Bcr expression and the presence of Bcr in postmitotic neurons of the hippocampus suggests that Bcr functions also in the absence of neuronal proliferation.

Postmitotic neurons in *bcr* expressing regions, the olfactory bulb, cortex, and hippocampal formation undergo intensive activity-related alteration of synaptic efficacy. It is intriguing to consider a role for Bcr in energy-consuming processes underlying cellular mechanisms of synaptic plasticity. We are currently examining this hypothesis by measuring neuronal Bcr at baseline conditions and subsequent to physiological stress.

The *C*-terminal part of Bcr has a high degree of homology with that of n-chimerin, which, like Bcr, possesses GTP-ase activity toward p21*rac*, a member of the RAS superfamily. The

regional expression pattern of *bcr* in the brain overlaps in part that of n-chimerin. *N-chimerin* expression is restricted to neurons, with the highest levels of expression in hippocampal pyramidal cells, granulae cells of the dentate gyrus, in cortical neurons, and in the Purkinje cells of the cerebellum (Lim et al., 1992). The developmental expression of *bcr,* however, displays different dynamics than that of *n-chimerin*: whereas *n-chimerin* expression rises sharply after birth to maximal levels at 20 d postpartum (Lim et al., 1992), a period coinciding with cellular differentiation and synaptogenesis, *bcr* expression remained constant from E15 through day 35 after birth. Northern blot analysis of hematopoietic organs (liver and spleen) also revealed a constant level of *bcr* expression from embryonal stage E15 throughout development until adulthood.

It is difficult to assess the significance of the relatively high level of *bcr* expression in the hippocampal region of the brain. Recently, n-chimerin expression in the canary forebrain was connected with seasonal changes in song learning and behavior; the conserved structure and pattern of n-chimerin expression in the song circuit suggested a fundamental function for this gene in the vertebrate forebrain and a role in the regulation of neural plasticity (George and Clayton, 1992). In the young mouse brain, the developmental expression pattern of *n-chimerin* seems to disclose a potential role in the development of particular cognitive functions (Lim et al., 1992). The finding that *bcr* expression is more or less constant throughout brain maturation would seem to point to a regulatory role of a more fundamental character in cellular processes.

In the rodent brain, *bcr* mRNA in the hippocampus is most abundant in dentate gyrus cells, lower in CA3, and lowest in CA1. The functional organization of the hippocampus is such that input through the perforant path reaches granule cell layers. The granular cells, through axons (mossy fibers), synapse on CA3 neurons, which then impinge on CA1. The gradient of *bcr* expression levels along this functional neuronal path may reflect a potentially interesting yet presently poorly understood function of Bcr in these highly specialized structures.

A growing number of GTPases and GAPs has been implicated in brain biology over the most recent years. Often these proteins (i.e. n-chimerin, α2-chimerin, Bcr, Abr) display a brain-specific expression pattern (Hall et al., 1993; Leung et al., 1994; Lim et al., 1992; Tan et al., 1993) or are, as described above, implicated in brain physiology (n-chimerin) or neoplasia (neurofibromin). Members of the extended family of GTPases have also been implicated in synaptic vesicle transport in nerve terminals (Liu et al., 1994; Von Gersdorff and Matthews, 1994), further indicating the importance of such protein factors in fundamental brain-related processes. This study, concurrent with the recently demonstrated function of Bcr as a regulator of p21*Rac*-mediated superoxide production by the respiratory burst oxidase in neutrophils (Voncken et al., 1995), suggests differences in tissue-specific functioning of p160*Bcr*. Increased knowledge of these cellular function(s) of Bcr should strengthen our understanding of the role of Bcr in leukocytes, its contribution to *Ph*-positive leukemogenesis, and its biological relevance in the brain.

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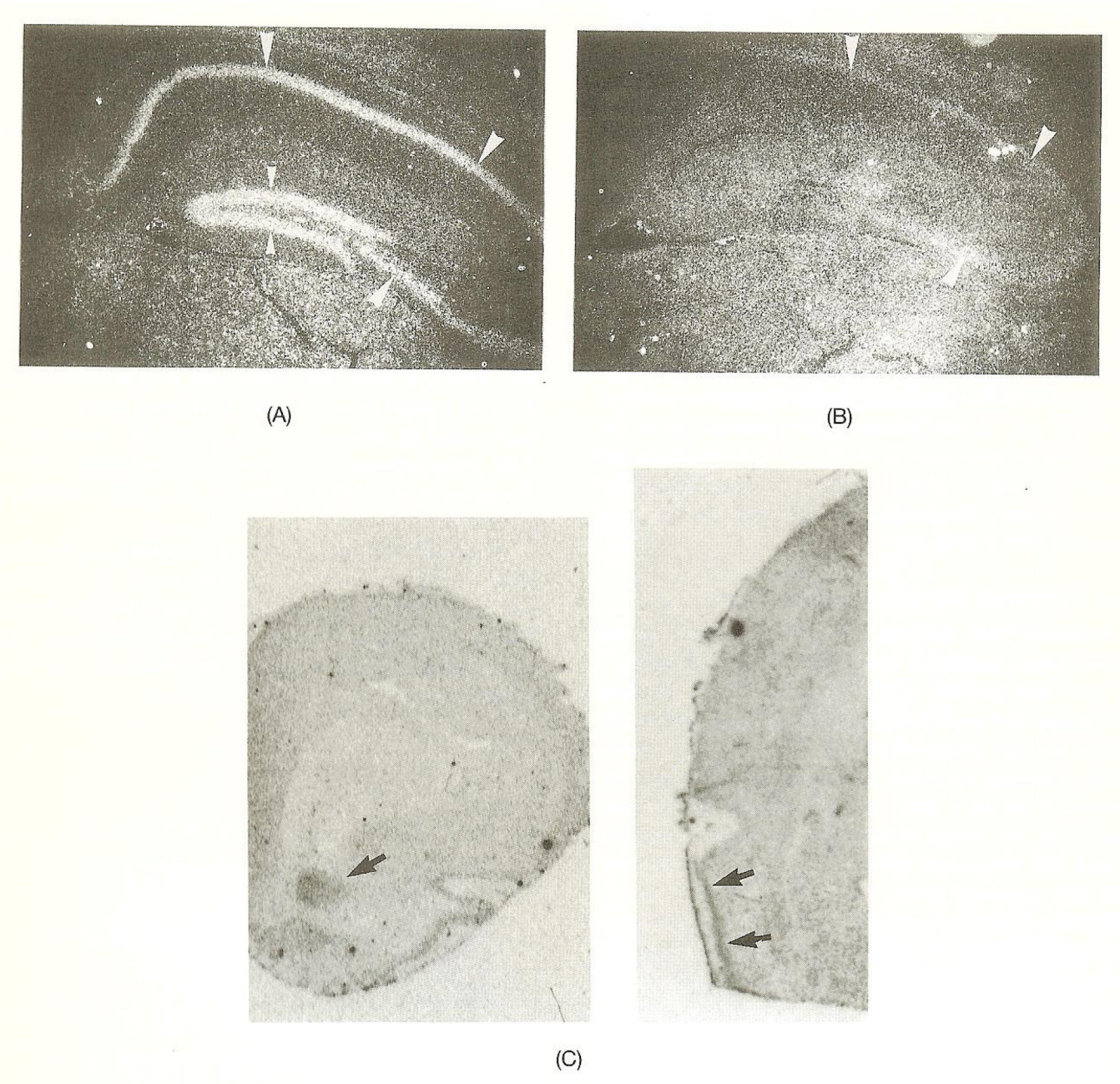

Fig. 1.

(A) Darkfield photograph of a coronal section of a mouse brain showing strong hybridization to the hippocampus (large arrow-heads) and dentate gyrus (small arrow-heads) when hybridized wilh the antisense *her* probe. (B) Daikfield photograph of a parallel section to the one presented in Fig. IA showing only weak background hybridization to the hippocampus (large arrowheads) and no hybridization signal to the dentate gyrus when hybridized with the sense *bcr* probe. (C) Magnifications of rat olfactory bulb (to the left) and whole brain sections (to the right) showing specific hybridization of a bcr-specific oligonucleotide to anterior olfactory nucleus and piriform cortex (arrows). Autoradiographic image.

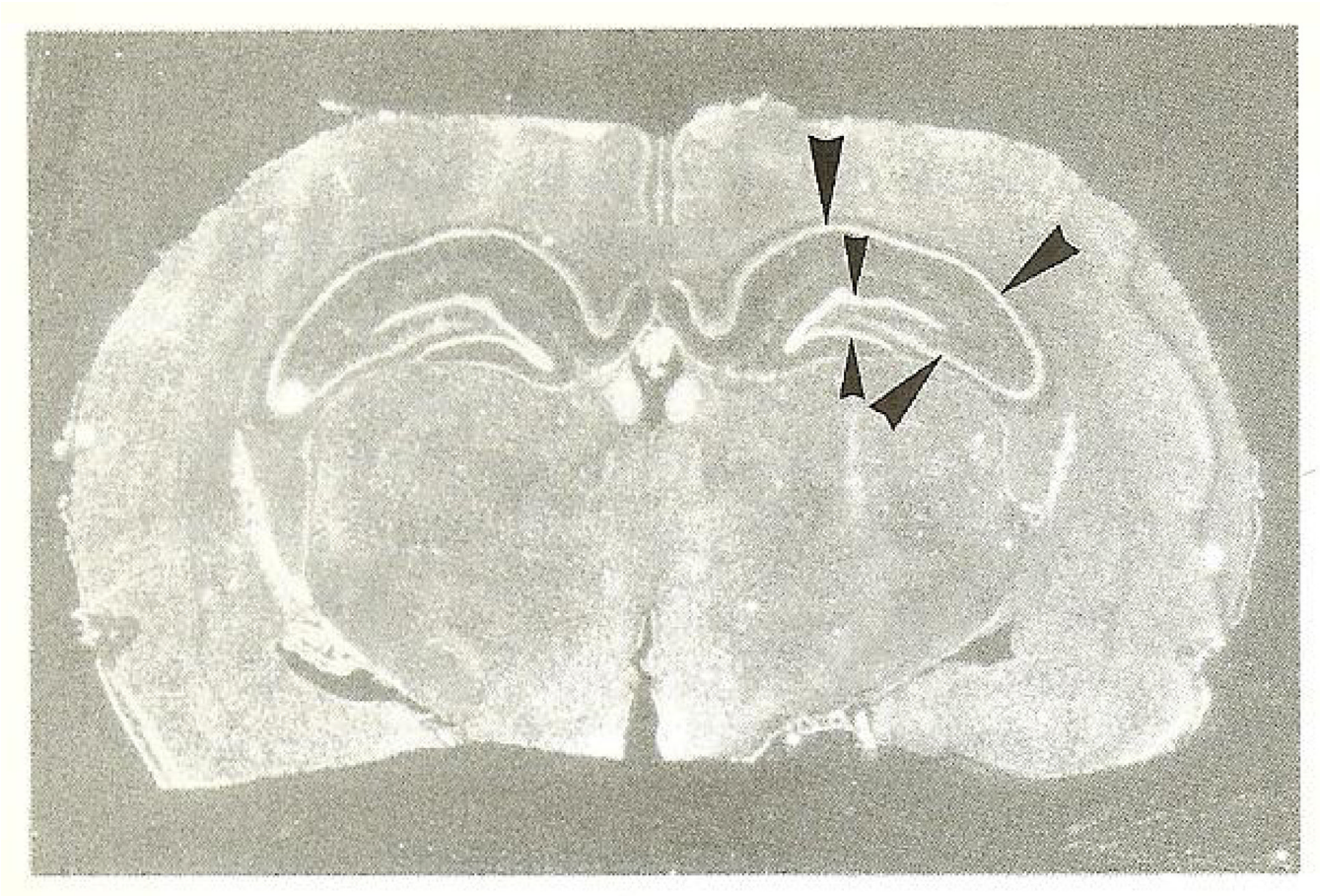

Fig. 2.

Coronal section of the rat brain showing strong hybridization to the hippocampus (large arrow-heads) and dentate gyms (small arrow-heads) when hybridized to the *bcr* oligonucleotide (see the Material and Methods section). Reversed autoradiographie image.

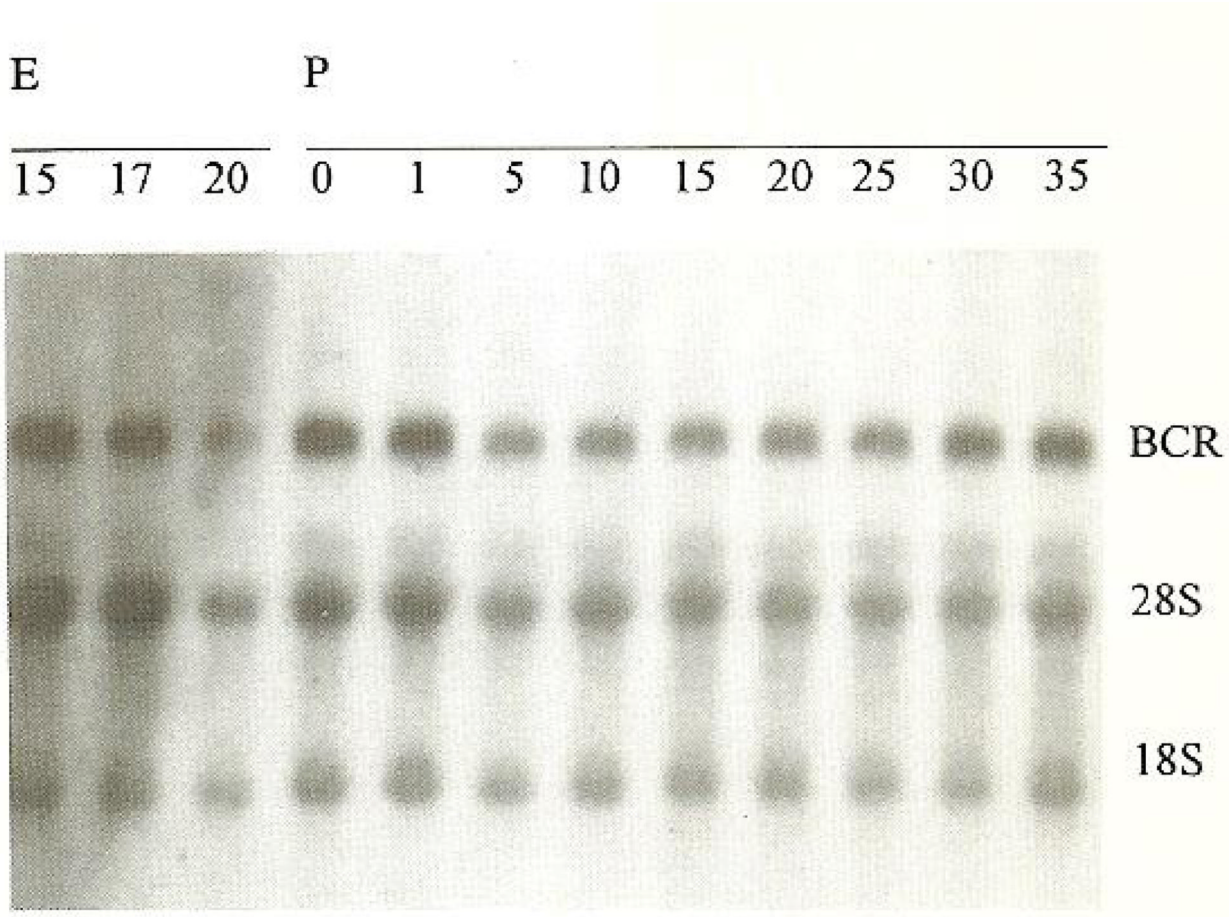

Fig. 3.

Autoradiogram showing a constant expression of *bcr* in the mouse brain from embryonal day 15 (E15) to postnatal day 35 (P35). The *bcr* transcript of approximately 7 kb and the 28 and 18S ribosomal bands are indicated.