Brain "Identifier Sequence" Is Not Restricted to Brain: Similar Abundance in Nuclear RNA of Other Organs

Abstract. *A repeated 82 base pair sequence in genomic DNA of the rat was previously proposed as being a control element governing brain (neuron) specific genetic expression. This intronic sequence, termed the brain "identifier" (ID), is complementary to small RNA species localized in brain cytoplasm, and it was thought to be represented specifically in RNA produced by brain nuclei in vitro. The RNA blot analyses of total nuclear and polyadenylated heterogeneous nuclear RNA described in the present report show that this ID sequence is also present in the liver and kidney in abundances similar to those in the brain. This repeated sequence is not, therefore, restricted to transcripts produced in the brain as suggested from previous transcriptional "runoff" experiments. Measurements on rat and mouse nuclear RNA indicate that the abundance of ID sequence transcript is roughly proportional to the number of copies of this repeat in the respective genomes. This suggests a rather random genomic location and transcription of this sequence. From these results it seems improbable that the ID sequence functions as a transcriptional-level control element in genes expressed specifically in the brain.*

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A repeated 82 nucleotide sequence (copy frequency estimated at $\sim 1.5 \times 10^5$ copies in the rat genome) has been described as a component of primary transcripts and assorted small RNA molecules uniquely present in the brain (*1–3*). This repeat has been termed the brain "identifier" (ID) sequence (*1*). The ID sequence occurs in the introns of several genes such as the rat growth hormone gene (*4*) and in the introns of certain genes which appear to be expressed specifically in the brain (*2*). The ID contains a consensus sequence for initiation of transcription by polymerase III, and transcription by this polymerase is thought to yield small RNA species (~110 to 160 nucleotides) which appear in the cytoplasmic fraction of the brain, but not in other organs such as the liver or kidney (*3*). Sutcliffe *et al.* found from transcriptional "runoff" experiments that 62 percent (normalized for recovery efficiency) of the mass of radioisotopically labeled transcript produced by brain nuclei contained ID sequence, whereas ID sequence containing transcripts from liver and kidney nuclei were apparently absent because measurement above a "background level" of the assay was not detected (*3*). Electrophoretic analysis of the ID-sequence–containing RNA from brain nuclei showed the presence of a species of ~110 nucleotides, a smear of molecules somewhat above and below 500 nucleotides, and unresolved, labeled material which remained in or near the application well of the electrophoretic gel. The latter unresolved material was considered to be large, heterogeneous transcripts produced by polymerase II, because synthesis was reduced in the presence of α-amanitin (*3*). Sutcliffe *et al.* concluded that "ID sequences are located in a substantial portion of brain transcripts synthesized by both polymerase II and polymerase III but are missing from liver and kidney transcripts" (*5, 6*). From these and other observations an interesting model was proposed in which ID sequences function as controlling elements of brain-specific genetic expression (*5*).

We used a cloned DNA [cDNA; clone p2A120, see (*1*)] containing the ID sequence to probe total nuclear RNA and polyadenylated [poly(A)$^+$] heterogeneous nuclear RNA (hnRNA) from the brain, liver, and kidney of the rat. Our results show that the ID sequence is present in similar abundance in the nuclear RNA from all of these organs. For mouse nuclear RNA's a similar result was obtained, although in this species ID transcript abundance is less than in the rat. Hence, as measured in nuclear RNA the ID sequence appears not to have any special qualitative or quantitative specificity pertaining to genetic transcripts in the brain or in other organs.

In a dot blot series of decreasing amounts of RNA, a proportionate decline in the radiographic signal was obtained for all RNA's (Fig. 1A). Dilution series have been shown to provide quantitative estimates of the relative abundance of specific species in complex RNA populations (*7, 8*). Hybridization of the ID probe is attributable to RNA as shown by the elimination of hybridization when RNA (but not DNA) samples were treated with alkali (Fig. 1A). Measurement by scintillation counting of triplicate dot blot filters (Fig. 1B) showed that the amount of hybridized ID sequences DNA, as visualized radioautographically, was similar in all blots of nuclear RNA. In several other experiments of this type, the amount of ID sequence DNA which hybridized was always within a factor of 2 for all of the nuclear RNA's.

We considered the possibility that ID sequences, although present in liver and kidney nuclear RNA, might be restricted to the brain in terms of poly(A)$^+$ hnRNA, the fraction of RNA containing the precursors of poly(A)$^+$ messenger RNA (mRNA). This possibility was eliminated by RNA dot blot analyses of poly(A)$^+$ hnRNA from each of the organs which showed that the ID sequence is present in roughly similar abundance in all these RNA's (Fig. 1C) (*9*).

The model of tissue specific genetic regulation offered by Sutcliffe *et al.* (*5*) is, in part, based on the contention that the primary transcripts of structural genes expressed specifically in the brain contain the ID sequence. Therefore, we measured by Northern blot analysis the approximate size of the poly(A)$^+$ hnRNA molecules that contain sequences complementary to the ID probe. As shown in Fig. 1C, the ID probe hybridized to molecules of diverse size, most of them in the range of 2,000 to 11,000 nucleotides (*10*). Thus, poly(A)$^+$ hnRNA molecules in the size range characteristic of many mRNA precursor molecules contain sequences complementary to the ID probe. Again, in these analyses, no apparent specificity with respect to brain was observed because the probe hybridized to a broad smear of hnRNA molecules in all cases.

Because a high degree of homology or family-like relationship exists between the ID repeat and another repeated sequence isolated from a rat genomic DNA which hybridizes with nuclear RNA from liver, kidney, and neural crest–derived PC12 cells (*11*), we asked whether the quality of all the hybrids was the same. On the basis of a number of physical parameters (*12*), we estimated that nearly complete dissociation should occur at ~75°C in the presence of low concentrations of sodium ions [0.03*M* or 0.2× standard saline citrate (SSC)]. Hybrids (dot blots) were taken through a wash series in 3°C increments starting at 67°C. Dissociation was the same for all hybrids. The same relative amounts of hybridized ID probe remained in association with each of the RNA's at 73°C, and >90 percent dissociation was observed at 75°C in all cases. Hence, the base pairing fidelity, based upon thermal stability, is the same for hybrids formed between the ID sequence and all of the nuclear RNA's.

The cDNA clone p2A120 also contains a sequence complementary to a putatively brain-specific RNA species (*2*). Despite this putative specificity, this portion of the insert might have contributed to, or accounted for, the hybridization we observed with liver and kidney RNA's. To verify that the hybridization signal in nuclear RNA was due exclusively or largely to the ID sequence, we removed it from the plasmid DNA. Restriction sites exist which permit the isolation of the last 65 bases of the 82 base repeat plus oligoadenylate and oligocytidylate segments of 20 and 11 nucleotides respectively [see reference (*1*) for sequence and restriction map]. We purified this shortened "pure" ID sequence and used it to probe nuclear and poly(A)$^+$ hnRNA. With this fragment, dot and Northern blot hybridization results were similar to the result when the entire p2A120 plasmid DNA was used as the probe (Fig. 1D). When nuclear RNA was hybridized with restriction fragments of pBR322 DNA, or non-ID portions of the cDNA insert, a signal was not observed (1 to 2 μg of RNA blotted).

The number of ID sequences in the rat genome might be fivefold greater than the number of structural genes that are thought to be expressed in a brain-specific manner (*5*), although this is speculative as the average number of these repeats per transcriptional unit is unknown. If this is the case, perhaps transcription of these intron-located sequences might often occur in a wide variety of cells in accordance with a random distribution within the genome, thus obscuring any possible brain-specific aspect of ID sequence transcription. However, in the mouse genome the copy number of the ID sequence is about sevenfold less than in the rat, $\sim 1.9 \times 10^4$ compared to $\sim 1.3 \times 10^5$ copies (*13*, *14*). If this lower copy number is due to a more specific distribution of the ID sequence within the mouse genome, perhaps to the extent that it is restricted largely or completely to genes that are expressed specifically in the brain, then brain-specific quantitative differences should be readily observed in this species. Therefore, we measured ID sequence transcripts in organs from the mouse as done for the rat. As shown in Fig. 1E, the ID sequence is present in roughly similar abundance in all of the mouse nuclear RNA's, and, as shown by a Northern blot of liver nuclear RNA, it is present in molecules of diverse size as in the rat. Northern blots of brain and kidney nuclear RNA's showed a similar molecular weight distribution of ID sequence–containing molecules. Hence, as in the rat, ID sequence representation is neither restricted to, nor enriched in, transcripts produced in the brain. Instead, the results show transcription is extensive in widely different organs suggestive of the possibility that transcription occurs in accordance with a rather random intronic distribution of the sequence within the genome. Indeed, in rat liver nuclear RNA the repeat is about five times more abundant than it is in mouse liver nuclear RNA (Fig. 1E). Hence the abundance of this repeat in nuclear RNA of the rat and mouse is roughly proportional to the copy number of the sequence in the respective genomes. This is consistent with the notion of random intronic distribution and transcription of this repeat.

While these results show that the 82 base pair repeat is represented to a similar extent in the nuclear RNA from widely different complex organs, and thus it appears not to meet the qualitative requirements of a genetic control element functional at the transcriptional level uniquely in brain cells, a regulatory role for this sequence remains possible. Sutcliffe *et al.* (*1*) have shown that a 160 nucleotide brain cytoplasmic RNA spe-

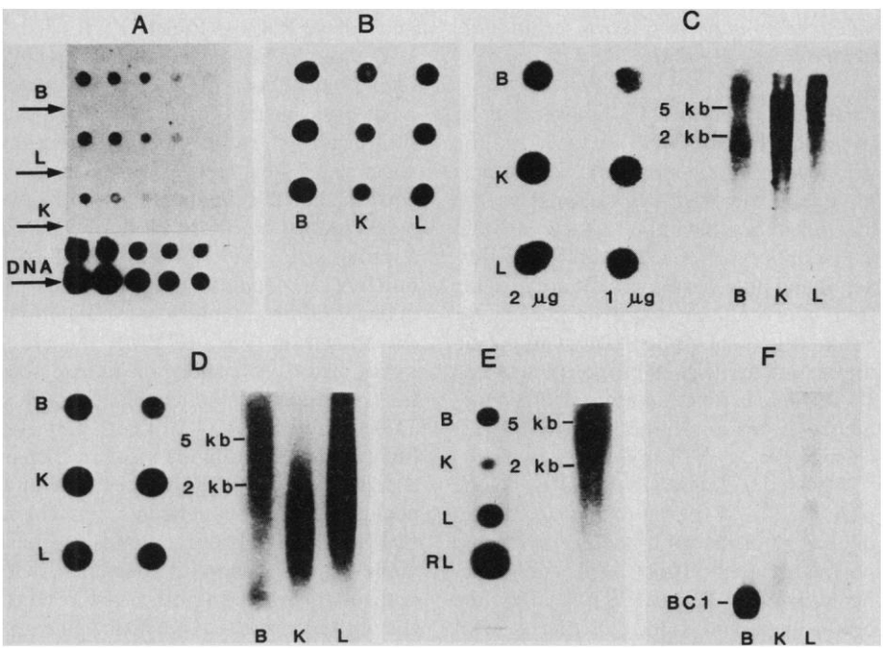

Fig. 1. Hybridization of ID plasmid p2A120 DNA, or "pure" ID probe, to total nuclear, poly(A)$^+$ hnRNA, or poly(A)$^+$ cytoplasmic RNA from rodent brain, liver, and kidney. Experimental details are described (*24*). (A) Hybridization of ID-containing plasmid DNA (p2A120) to nuclear RNA. Twofold serial dilutions of 2 μg of brain (B), liver (L), and kidney (K) total nuclear RNA, or 5 ng of pBR322 DNA blotted onto nitrocellulose filters were hybridized with plasmid p2A120 DNA. Samples in rows indicated by arrows were treated with 0.2*M* NaOH for 2 hours at 67°C and then neutralized before being blotted. (B) Quantitation of hybridization of ID-containing plasmid DNA to nuclear RNA. Triplicate samples (2 μg) of brain (B), kidney (K), and liver (L) total nuclear RNA were hybridized with plasmid p2A120 DNA. After autoradiography, individual blots were cut from the filter and the radioactivity was determined by scintillation counting. The average radioactivity, expressed as counts per minute, and after correction for background (39 ± 3), was: brain, 177 ± 40; liver, 185 ± 27; kidney, 115 ± 8). (C) Dot and Northern blots showing hybridization of ID-containing plasmid DNA to poly(A)$^+$ hnRNA. Dot blots were done with the indicated quantities of poly(A)$^+$ hnRNA from brain, kidney, and liver. In Northern blot analysis, each lane contained ~2 μg of brain, kidney, or liver poly(A)$^+$ hnRNA. (D) Hybridization of ID probe removed from plasmid DNA by restriction endonucleases ["pure" ID, see (24)] to poly(A)$^+$ hnRNA. The poly(A)$^+$ hnRNA is the same as in the dot blots shown in Fig. 1C which, after removal of the p2A120 probe, was rehybridized with end-labeled ID-specific probe. For Northern blots, ~1 μg of poly(A)$^+$ hnRNA from either brain, kidney, or liver was subjected to electrophoresis in each lane and hybridized to ID-specific probe. Kidney RNA used in this experiment was slightly cleaved, probably because of endogenous ribonuclease, a common problem with this organ (compare with C). (E) Nuclear RNA (4 μg) from mouse brain, liver, and kidney hybridized with rat ID sequence DNA. Quantitation, in counts per minute, of hybridized DNA determined by scintillation counting of triplicate blots was: brain, 174 ± 25; liver, 258 ± 25; and kidney, 104 ± 16. By comparison, about five times more DNA hybridized to a like amount of rat liver (RL) nuclear RNA (1200 ± 128 count/min) than to mouse liver RNA. Final wash conditions for the RNA dot blots were 0.2× SSC at 60°C. The blot of electrophoretically separated mouse liver nuclear RNA shows a size distribution of ID sequence-containing molecules similar to that observed in poly(A)$^+$ hnRNA from rat organs, see (C). (F) Northern blots of cytoplasmic poly(A)$^+$ RNA hybridized with "pure" ID DNA. Each lane contained ~1 μg of poly(A)$^+$ cytoplasmic RNA from brain, kidney, or liver.

cies (BC1-RNA) complementary to the ID sequence is present in brain, but absent in other organs such as liver and kidney. As shown in Fig. 1F, we confirm that the ~160 nucleotide RNA species is very evident in poly(A)$^+$ cytoplasmic RNA of the brain, but not in liver and kidney (*15*). In the latter organs, hybridization is observed as a faint smear throughout the blot. While no functional role of this brain cytoplasmic 160 nucleotide species has been shown experimentally, it might serve as a signal in selective transport via attachment to brain-specific mRNA-protein complexes, being freed from the complex once transport is complete. Other possibilities for this RNA might be in modulation of translation of mRNA or as a component of a signal recognition particle, perhaps analogous to 7 SL RNA (*16*).

The observation that the copy number of this sequence in mouse and rat varies by nearly an order of magnitude seems to be inconsistent with a regulatory role in the selective expression of genes (*17*). It seems that the copy number of an important transcriptional control element would be conserved relative to the number required for function, because the probability of deleterious location of such a sequence increases with copy number. Thus, while this 82 base pair repeat and respective small RNA's might have a control function, a role as a control element in governing brain specific gene expression at the transcriptional level seems improbable.

References and Notes

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9. In our experiments we often observed somewhat less hybridization with brain poly(A)$^+$ hnRNA than with liver or kidney. If one assumes the extent of cleavage is random and similar in all preparations, this suggests that ID sequence-containing poly(A)$^+$ hnRNA might be processed and transported somewhat more rapidly in brain such that steady-state levels of this RNA are lower than in liver or kidney. More extensive measurements are required to determine this possibility.
10. The size determination of poly(A)$^+$ hnRNA hybridizing with ID sequences was based on the migration distances in these gels of rat 28*S* ribosomal RNA (~5000 nucleotides), 18*S* ribosomal RNA (~2000 nucleotides), and transfer RNA (~80 nucleotides). Since no RNA marker larger than 5 kilobases was used, the upper size limit of hybridizing transcripts was estimated by assuming the rate of migration of denatured RNA to be linearly proportional to $\log_{10}$ of the molecular weight. The distribution of molecules reacting with the probe does not necessarily represent the average steady-state size of poly(A)$^+$ hnRNA. Some of the smaller RNA molecules hybridizing with the probe might be fragments formed by degradation of larger molecules during purification and fractionation.
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15. Similar results with brain cytoplasmic RNA were obtained by J. Brosius (personal communication). Brosius did not detect BC1 RNA in Northern blots of brain polysomal RNA. Sutcliffe *et al.* (*1*) also obtained a slight, broad smear of hybridization in some blots of kidney and liver poly(A)$^+$ RNA probed with ID sequence DNA. In our experiments we suspect the slight hybridization smear might be due to nuclear RNA which contaminates the cytoplasmic fraction more so in liver and kidney than in preparations from brain.
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24. Total nuclear RNA was extracted from isolated nuclei (*18*) in the presence of 10 m*M* vanadyl-ribonucleoside complex by the hot phenol method at *p*H 5.2 (*19*). In experiments with total nuclear RNA, samples were first treated with deoxyribonuclease I (200 μg/ml) for 75′ at 37°C and the RNA repurified by extraction with phenol-chloroform and precipitation with ethanol. This procedure eliminated all exogenous pBR322 DNA that was added to a control sample of nuclear RNA prior to deoxyribonuclease digestion. For dot blotting, samples quantified by absorbance at 260 nm were denatured in a phosphate-buffered solution containing 50 percent formamide and 6 percent formaldehyde (*20*) for 20 minutes at 67°C, cooled on ice, diluted with 5 volumes of 20× SSC, and then applied onto wet nitrocellulose papers. In Northern gel analysis, the RNA was denatured as described above, fractionated by electrophoresis in 1.5 percent agarose gels in the presence of 2*M* formaldehyde (*20*), and then blotted onto nitrocellulose (*21*). Blots were baked for 2 hours at 80°C and hybridized overnight at 42°C in 50 percent formamide: 0.6*M* NaCl, 40 m*M* Na:PO_4 (*p*H 7.4), 4 m*M* EDTA, 0.1 percent SDS, herring sperm DNA (100 μg/ml), and 5× Denhardt's solution (*22*). Hybridizations were carried out for 24 to 48 hours at 42°C in fresh solution containing heat-denatured ^{32}P-labeled probe. The hybridization solutions used in D and F also contained poly(A) (1 μg/ml). Blots were washed at 42°C in three changes (at 15-minute intervals) of 2× SSC and 0.2 percent sodium dodecyl sulfate (SDS), and finally in 0.2× SSC and 0.2 percent SDS for 30 minutes at 42°C. In some experiments, an additional 30-minute wash in 0.2× SSC and 0.2 percent SDS at 67°C was performed. The hybridization in A to C was done with nick-translated cloned plasmid p2A120 DNA [for sequence and restriction map, see (*1*)], specific activity 0.5×10^8 to 2×10^8 cpm/μg. In D and F, a 93 base pair restriction fragment comprising the last 65 nucleotides of the ID sequence plus short oligo(A) and oligo(C) tracts was used as probe. This fragment was obtained by digesting p2A120 DNA with the restriction endonucleases Dde I and Pst I and isolating the appropriate sized fragment from an 8 percent acrylamide gel (*23*). Prior to electrophoresis, the 3′ recessed ends generated by Dde I digestion were labeled by filling in with α ^{32}P-labeled deoxyribonucleotides using the Klenow fragment of *E. coli* DNA polymerase I (*23*).
25. We thank J. G. Sutcliffe for plasmid p2A120 DNA. Supported by NIH grant NS10813.

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Superinduction of c-*fos* by Nerve Growth Factor in the Presence of Peripherally Active Benzodiazepines

Abstract. *Alterations in proto-oncogene expression after stimulation of rat pheochromocytoma (PC12) cells by nerve growth factor (NGF) have been investigated. A specific stimulation of* c-fos *messenger RNA and protein was detected 30 minutes after treatment. This induction was enhanced more than 100-fold in the presence of peripherally active benzodiazepines. The effect was specific as very little change was observed in the levels of* c-ras^{Ha}, c-ras^{Ki}, *c-*myc, *and N-*myc *messenger RNA's. Under the conditions used here, NGF treatment ultimately results in neurite outgrowth, with a reduction or cessation of cell division. Thus, stimulation of the* c-fos *gene in this system appeared to be associated with differentiation and not with cellular proliferation. The effect of benzodiazepines was stereospecific and represents a novel action of these compounds at the level of gene expression.*

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Neoplastic transformation is a consequence of the subversion of normal growth regulatory mechanisms. Furthermore, several retroviral oncogenes and their cellular progenitors are intimately associated with the normal processes of proliferation and differentiation. Many oncogene products share sequence homology with growth factors and their receptors (*1*), while expression of the c-*myc* and c-*fos* proto-oncogenes is stimu-