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Molecular characterization of the MT-family of dispersed middle-repetitive DNA in rodent genomes

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ABSTRACT

We report the consensus sequence of members of a new dispersed middle-repetitive DNA family, MT, which is present in mouse and rat genomes. This family is shown to be as abundant as the described rodent B1 and B2 families. Hybridization experiments with radioactive single-stranded cDNAs from different tissues indicate that MT sequences are more abundantly cotranscribed in parts of the brain than other repetitive families.

INTRODUCTION

Recently several interspersed repetitive DNA families have been described and well characterized. These include the primate LINE (1-6) and AluI (7) families and the rodent L1Md (8,9), B1, and B2 (10,11) families. These sequences represent a considerable amount of their genomes and have been isolated either as prominent bands of genomic DNA after restriction analyses (e.g. KpnI, AluI, MIF, E24, Bam5, R; see refs. 1,7,12-14) or as partial fold-back RNA by gradient analysis (B1, B2; see refs. 10,11). Conclusions drawn from these data concerning the evolutionary significance and modes of amplification and possible function have been reviewed (1,2,15,16).

We report here the molecular organization of an additional interspersed middle-repetitive DNA family present in rodent genomes with copy numbers comparable to B1 and B2 (10,11). Members of this family, MT (Mouse Transcript), have been isolated by random Southern blot analysis of several cDNA clones constructed from poly(A)$^+$RNA of cerebellar mouse tissue (17) followed by hybridization assays with known repetitive DNA sequences as probes and isolation of homologous clones from a M13 genomic mouse library. Sequence analyses of such clones allowed the establishment of a MT consensus sequence. Members of the MT family were detected in the 5' region of rat cytochrome P450 oxidoreductase gene (18) and in the mouse Ins sequence (19).

MATERIALS AND METHODS

Isolation and subcloning of cDNA clone CebA-847

CebA-847 was isolated from a cDNA-library prepared from poly(A)$^+$RNA of adult mouse cerebellum (17). The single-copy and repetitive portions of the 1250 bp recombinant DNA were separated and identified by Northern and Southern blot analyses (20,21). Repetitive sequences were detected by hybridizing CebA-847 restriction fragments with nick-translated (22) total genomic mouse DNA (23). Subfragments of the pcD-clone (24) were cloned into M13 vectors for sequencing or into pSP6-4 (38) for the use in hybridization experiments.

Isolation and subcloning of MT clones

Genomic clones homologous to the repetitive portion of CebA-847 were isolated from a genomic M13 sublibrary. Mouse DNA was digested with HindIII and BamHI, and ligated to HindIII/BamHI-cut M13mp18 (25). Recombinant phage were screened by plaque hybridization using the nick-translated (22) EcoRI/AvaII-fragment of CebA-847 (nucs 728-1111) as a probe. Thirty-six positive clones (MT1-MT36) were isolated, three of them were subjected to sequence analysis (see below).

DNA sequencing

All sequence data were obtained by the enzymatic chain terminator method (26) using subfragments cloned into M13mp18 or mp19 (25) templates. Five different subclones were used for the complete sequencing of CebA-847: 1 and 2. a 728 bp PstI/EcoRI-fragment in both orientations, 3. a 600 bp EcoRI/BamHI-fragment (using an internal EcoRI-site and the BamHI-site in pcD) in mp19, 4. a 517 bp EcoRI/blunt end-fragment generated by limited digestion of the 728 bp PstI/EcoRI-fragment with nuclease Bal31 (27) and subcloned into EcoRI/HincII-cut mp18, 5. two BamHI/blunt end-fragments (300 and 470 bp) generated by Bal31 digestion of the 600 bp EcoRI/BamHI-fragment subcloned into BamHI/HincII-cut mp18. The genomic MT clones 4, 13, and 28 were chosen for sequence analysis. MT-4 (1.3 kb) and MT-28 (0.95 kb) were first sequenced on the initial mp18 templates and then subclones were constructed using sequenced restriction sites. A BamHI/HincII-clone of MT-4 and a HindIII/PvuII-clone of MT-28, both in mp18, were subjected to further sequence analysis. MT-13 (3.2 kb) was digested with HaeIII, the resulting fragments resolved on an agarose gel, transfered to nitrocellulose filters, and probed with the nick-translated CebA-847 probe as described above. The positive HaeIII-fragment (350 bp) was subcloned into SmaI-cut M13mp18 and sequenced.

Computer analysis

Sequence data were analyzed on the VAX/VMS system using software from the University of Wisconsin (28,29). All sequences were compared to the UWGCG and EMBL gene databanks.

DNA blotting and hybridization

Blots were hybridized with 5 x 10^7 cpm of nick-translated probe DNA at 42°C in 50% formamide / 5 x SSC / 5 x Denhardt's (30) / 100 µg/ml salmon sperm DNA / 100 µg/ml E.coli DNA / 25 µg/ml poly(U), washed twice in 2 x SSC / 0.1% SDS and twice in 0.1 x SSC / 0.1% SDS at 65°C.

Preparation of cytoplasmic poly(A)$^+$RNAs

Brain tissues, either total brain, cerebral cortex or cerebellum were homogenized in 5 volumes of ice-cold nucleus buffer containing 50 mM TrisHCl pH 7.5 / 25 mM NaCl / 15 mM $MgCl_2$ / 250 mM sucrose / 0.5% NP-40 / 10 mM vanadylribonucleoside complexes. The homogenate was centrifuged for 10 min at 1000 x g. The resulting supernatant was mixed with two volumes of homogenization buffer (5 M guanidinium thiocyanate / 50 mM TrisHCl pH 7.6 / 10 mM EDTA / o.1 M 2-mercaptoethanol). Then the protocol was followed as previously described (17).

Preparation of single-stranded ^{32}P-cDNA probes

One microgram of poly(A)$^+$RNA was mixed with 1 µg of oligo(dT) primer and reverse transcribed in 50 mM TrisHCl pH 8.3 containing 140 mM KCl, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 40 µCi of α-^{32}P-dCTP (>3000 Ci/mM), and 25 U AMV reverse transcriptase (Boehringer Mannheim). The incubation was carried out at 37°C for 60 min. Subsequently the probes were boiled for three minutes, chilled on ice, and digested with RNase A for 5 min at 37°C, before they were extracted twice with phenol and chloroform, and ethanol-precipitated.

Isolation and characterization of control clones

Repetitive control clones carrying sequences homologous to satellite DNA (31), L1 (8), B1 (10), and B2 (11), were isolated from the cDNA and genomic libraries by means of hybridization with nick-translated genomic DNA following sequence analysis. They were designated by RS-3 (satellite DNA), RS-13 and CebA-117 (L1, see ref. 17), CebN-B9 (B1), and CebA-C4 (B2). A full-length cDNA clone (CebA-D4) of mouse mitochondrial cytochrom oxidase II, isolated from the adult cerebellum cDNA-library on the basis of its hybridization to single-stranded cDNAs from different tissues at an equal rate, served as an internal control clone. Its identity was confirmed by DNA sequencing.

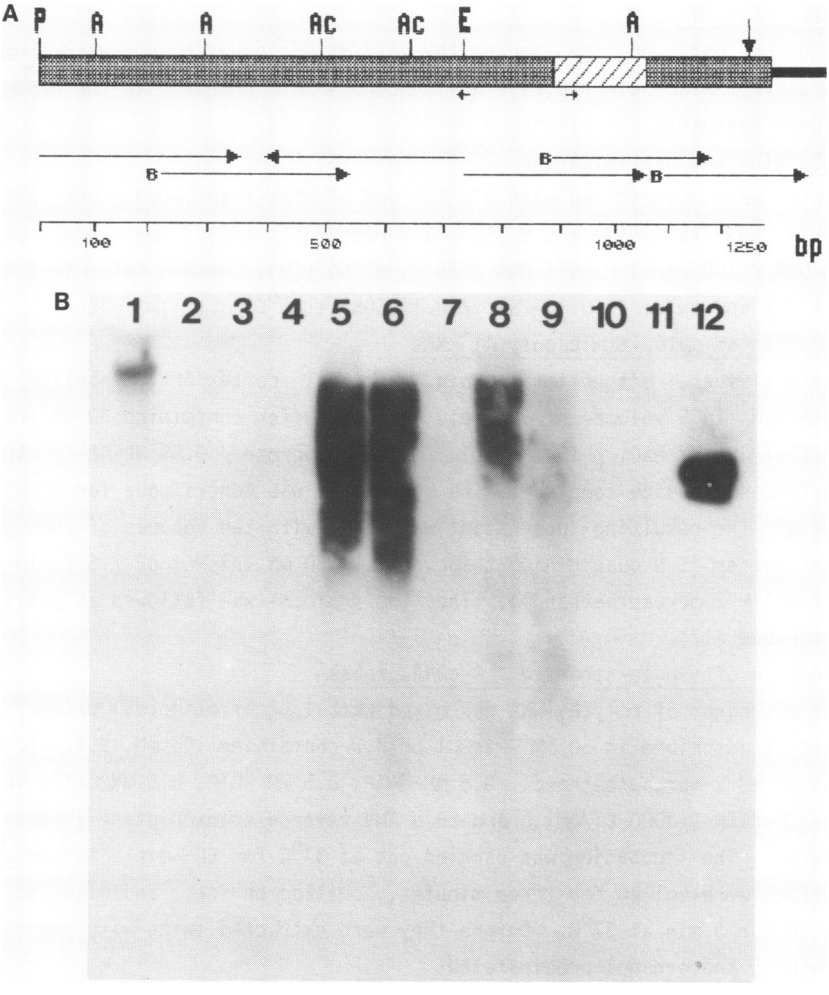

Fig. 1 A) Schematic diagram of the structure of cDNA clone CebA-847. P = PstI, A = AvaII, Ac = AccI, E = EcoRI. The black bar represents the poly(A) tail. Polyadanylation signal ATTAAA is indicated by a vertical arrow. MT sequences are hatched. The sequencing strategy is outlined by horizontal arrows. Arrows preceded by "B" represent Bal31 clones. Small horizontal arrows indicate positions of 11 bp inverted repeats (see text) B) Southern blot analysis on total genomic DNAs from different sources digested with EcoRI. Lane 1: E.coli RR1, lane 2: Z.mays, lane 3: D.hydei, lane 4: X.laevis, lane 5: C57BL/6J mouse brain, lane 6: mouse liver, lane 7: Lambda x HindIII, lane 8: LewOIa rat brain, lane 9: rat liver, lane 10: bovine heart, lane 11: human fetal liver, lane 12: mix of M13, SV40, and pBR322.

RESULTS

Identification of single-copy and repetitive portions of cDNA clone CebA-847

cDNA clone CebA-847 was isolated from a complete cDNA library which had been prepared from poly(A)$^+$RNA of adult C57BL/6J mouse cerebellum (17). Since this library was constructed using the pcD-system of Okayama and Berg (24), the clone starts with its relative 5' end at the vector-PstI-site followed by three G residues due to the G/C-tailing procedure. The insert is 1250 bp long from the PstI-site to the first residue of the poly(A)-tail. Initial Southern blots have indicated the presence of a dispersed repetitive element within the recombinant DNA. Its localization was determined more precisely by hybridization of nick-translated genomic mouse DNA to several restriction fragments (not shown). The smallest fragment labelled was an EcoRI/AvaII fragment (nucleotides 728 - 1111). Since the insert-EcoRI-site is unique, we used the 3' EcoRI fragment for Southern blot analysis on total genomic DNA (Fig. 1). A dispersed hybridization signal was visible only in lanes containing mouse and rat DNAs. However, the sequence analysis did not reveal any homology with previously described repetitive rodent DNAs, but revealed a unique 11 bp inverted repeat at nucleotide positions 710 and 946 (see Fig. 3 and discussion).

Isolation and sequencing of genomic MT clones

In order to determine more precisely the borders of the repetitive element, we screened (32) a M13 genomic sublibrary with nick-translated CebA-847 3'-EcoRI fragment. Thirty-six positive clones were isolated together with four colourless (i.e. hybridization-negative inserts) control clones. These forty single-stranded templates were primer-extended and cut with HaeIII. The resulting restriction patterns (Fig. 2) indicated that the MT-repetition is embedded in different neighborhoods of genomic sequences. Three clones, MT-4, MT-13, and MT-28, were selected for sequence analysis. A HindIII/PvuII-fragment of MT-28 (631 bp), a hybridization-positive HaeIII-fragment of MT-13 (316 bp), and two overlapping fragments of MT-4 (476 bp) have been sequenced. The sequencing strategies, restriction sites and homologous DNA stretches within these clones are shown in Fig. 3.

Homologies between different MT clones

Computer analysis of the determined nucleotide sequences allowed the establishment of a MT consensus sequence (Fig. 4). The relative 5' end (with respect to cDNA clone CebA-847) of the repetitive family was determined on the basis of three clones, CebA-847, MT-4, and MT-13Hae (see Fig. 3). This end of homology is located at nucleotide 887 of CebA-847. At the relative 3'

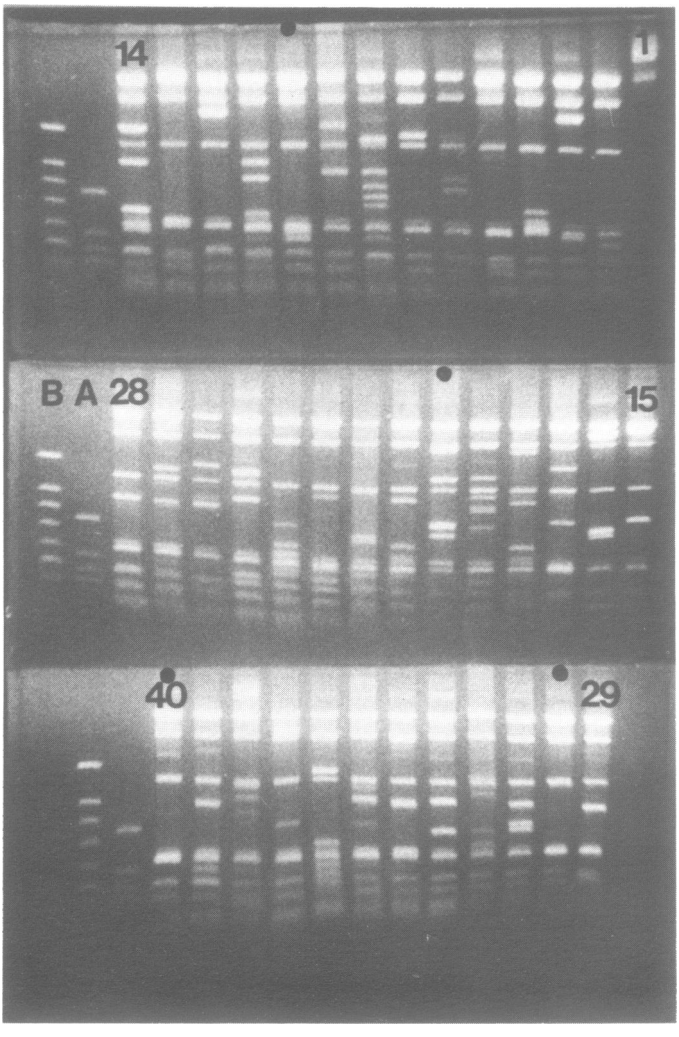

Fig. 2 HaeIII restriction patterns of genomic MT clones. Single-stranded, recombinant M13 templates were primer-extended, digested with HaeIII and resolved on a 1.5% agarose gel. Lanes 1 - 40: genomic MT clones, lanes A: CebA-847 insert digested with HaeIII, lanes B: size marker (3230, 1130, 910, 656, 521, 403, 280 bp). Black dots indicate colourless (i.e. hybridization-negative inserts) control clones MT-10, -20, -30, and -40.

end, however, the extension of the repetitive DNA is still unknown. Although MT-4 and MT-28 are not homologous to the extreme 3' end of CebA-847, they are homologous to each other (see Fig. 3). Additionally, a nick-translated HindIII/HincII-fragment of MT-4 (Fig. 3) hybridized to ten of the described

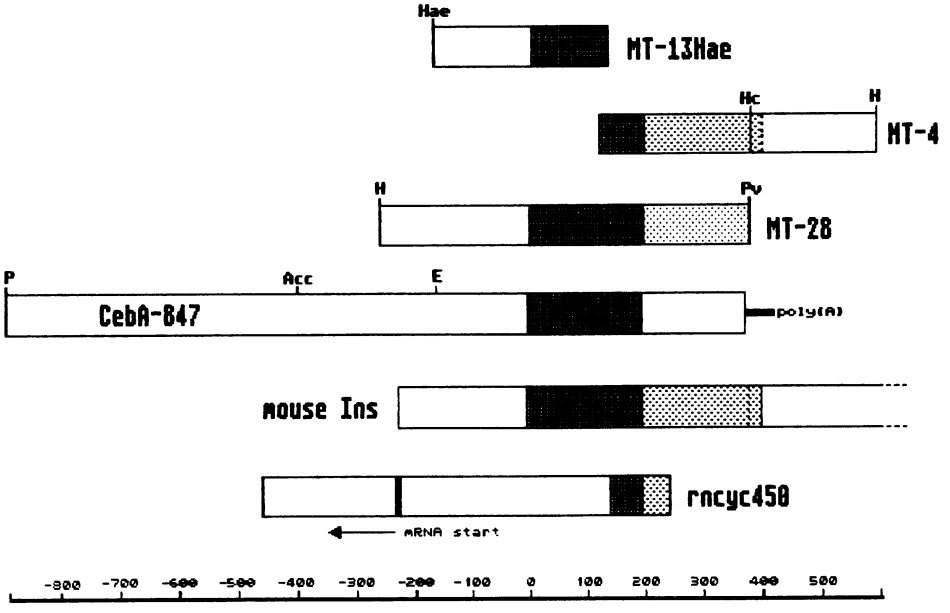

Fig. 3 Characterization of clones MT-4, MT-13Hae, MT-28, CebA-847, rat cytochrome P450 oxidoreductase (18) and mouse Ins DNA (19). Similarly hatched areas are homologous to each other (white areas = no homology). Hae = HaeIII, Hc = HincII, H = HindIII, Pv = PvuII, P = PstI, Acc = AccI, E = EcoRI.

36 genomic MT-clones (not shown). In confirmation of this result the homology between MT-4 and mouse Ins (19) extends further 25 bp downstream, where a B2 element is inserted into Ins (see Fig. 3).

Presence of MT sequences in the rat genome

As visualized by Southern blot analysis (Fig. 1), MT sequences are present in rat genomic DNA. This hybridization result was verified by computer comparison of MT sequences to the UWGCG and EMBL gene databanks. A member of the MT family was found to be present at the 5' region of rat cytochrome P450 oxidoreductase gene, 700 bp of which have been sequenced by Gonzalez and Kasper (18). The approximately 90% homology to MT sequences is shown in Fig. 4. The MT element starts 324 bp upstream of the mRNA cap site (see Fig. 3); the reletive 5' end, however, cannot be determined since the sequenced rat genomic clone begins only 429 bp upstream of the messenger start site.

Genomic copy number of MT sequences

In order to determine the copy number of MT sequences within mouse genomic DNA a hybridization experiment was performed which used immobilized DNAs of

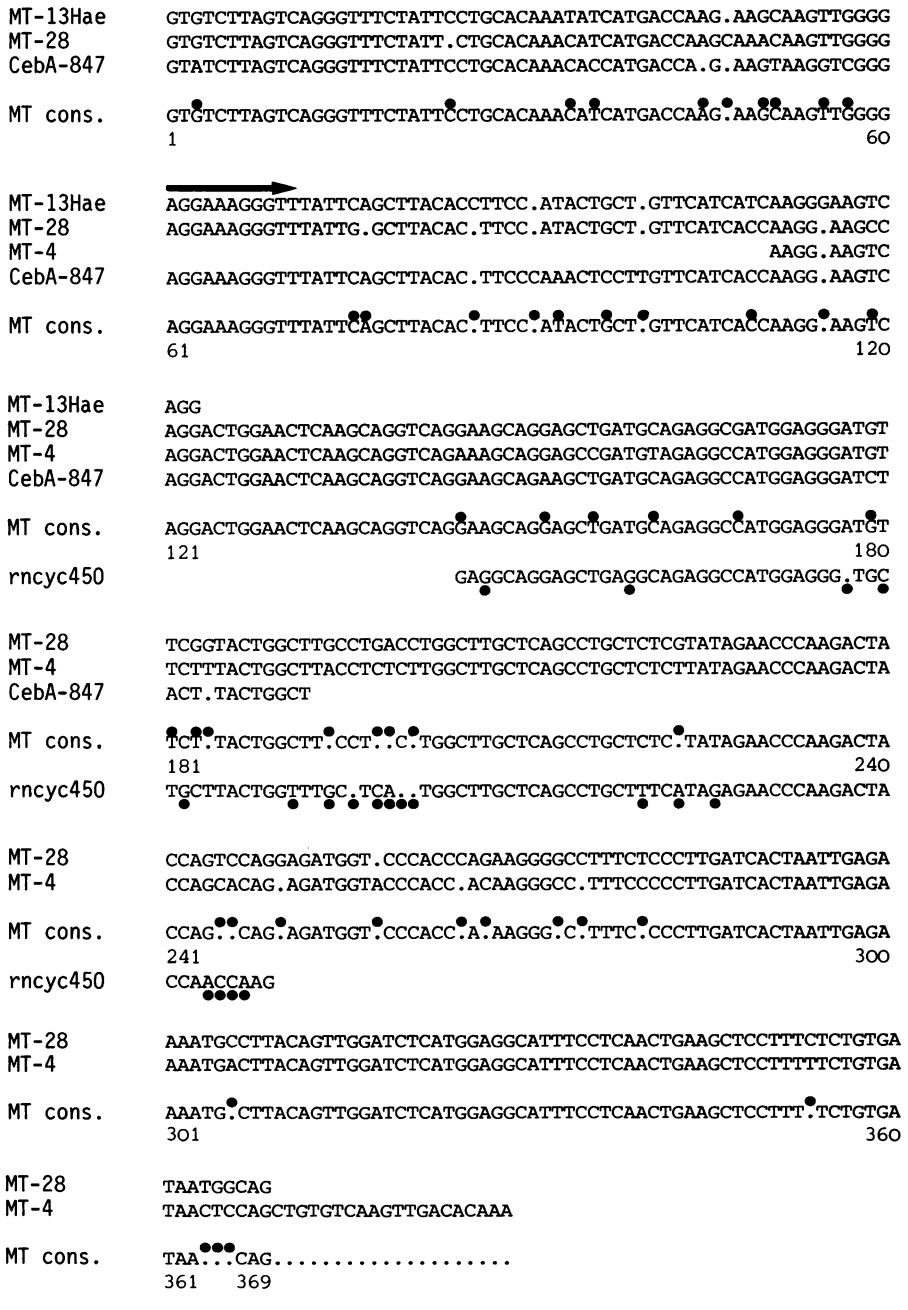

Fig. 4 Nucleotide sequence comparison of sequenced MT clones (see Fig. 3). Dots above the consensus sequence indicate mismatches. Arrow indicates the 11 bp repeat sequence within the consensus sequence (see Fig. 1A).

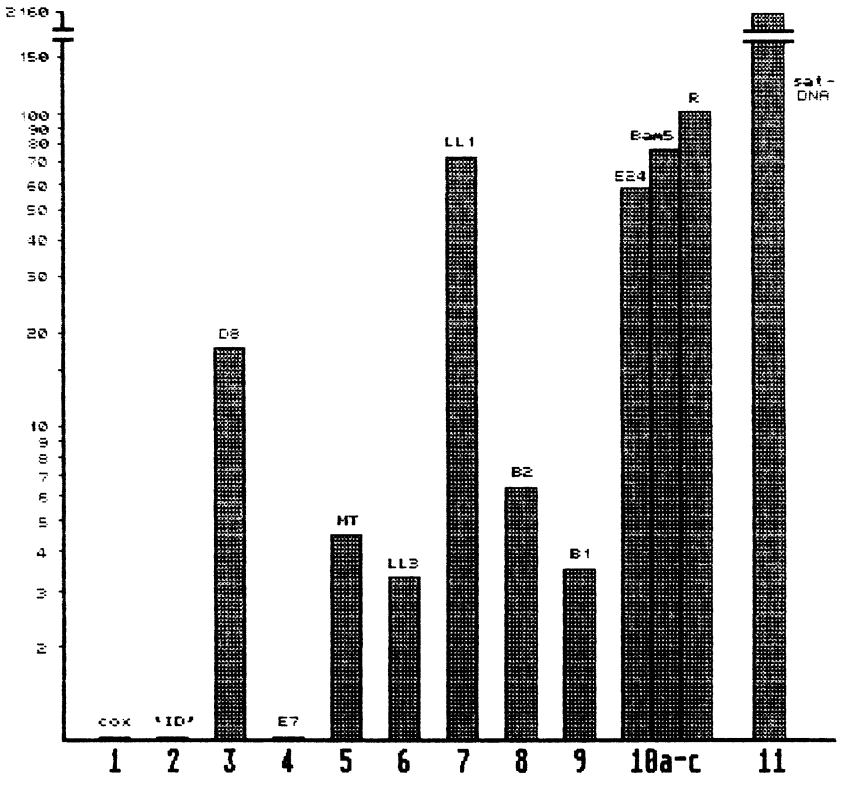

Fig. 5 Hybridization of radiolabeled total genomic mouse DNA to immobilized clones carrying repetitive sequences. The heights of bars correspond to the values of bound radioactivity measured densitometrically and are displayed on a logarithmic scale. 1: cytochrome c oxidase II, 2: R.dre.1 clone p2A120, 3: CebA-D8, 4: CebN-E7, 5: MT (CebA-847), 6: LLRep3, 7: LLRep1, 8: B2 (CebA-C4), 9: B1 (CebN-B9), 10 a-c: L1Md subsequences E27, Bam5, and R, respectively, 11: satellite DNA (RS-3).

several repetitive sequences including MT as targets and nick-translated genomic DNA as the probe. The resulting autoradiographs were scored densitometrically. The relative abundances are displayed as histograms in Fig. 5. In addition to MT-sequences the following control clones have been used (see legend of Fig. 5): 1. a full-length cDNA clone of mouse mitochondrial cytochrome oxidase II (33) isolated from our CebA-cDNA library; 2. clone p2A120 (34) which carries the rat repetitive family sequence R.dre.1; 3. CebA-cDNA clone D8, and4. CebN-cDNA clone E7, both carrying repetitive sequences not crosshybridizing with the other control clones; 5. MT; 6. LLRep3 and 7. LLRep1 (35); 8. CebA-cDNA clone C4 which carries a B2 family member; 9. CebN-

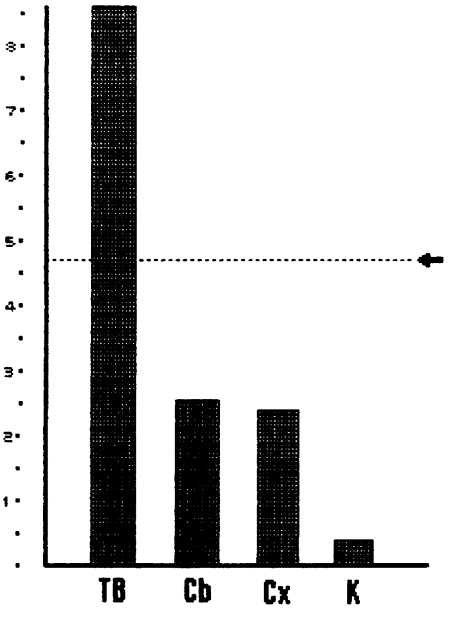

Fig. 6 Hybridization of single-stranded ^{32}P-cDNAs prepared from cytoplasmic poly(A)$^+$RNAs from different tissues. TB: total brain, Cx: cerebral cortex, Cb: cerebellum, K: kidney. The densitometric results obtained with cortex, cerebellum, and kidney cDNAs were correlated to the radioactivity bound by cytochrome c oxidase II-clone CebA-D4 from TB-cDNA (arrow).

cDNA clone B9 which carries a B1 repeat; 10 a-c. L1 family subclones carrying sequences homologous to E24 (36), Bam5 (13), and R (14). The subcloning of these three clones has been described previously (17); 11. genomic clone RS-3 consisting of sequences homologous (98%) to mouse satellite DNA (31) (all these sequence data and homology comparisons are not shown, but are available on request).

The extent of hybridization of radioactive genomic DNA was similar to that observed for both the B1- and B2-family sequences (see Fig. 5) and the copy number of MT sequences within mouse genomic DNA was determined as those reported for B1 and B2 (see refs. 10,11), i.e. between 4 and 9 x 10^4 per haploid genome.

Expression of MT sequences

The abundance of MT sequences on cytoplasmic poly(A)$^+$RNAs of several tissues (especially parts of the mouse brain) was determined by a similar experiment as described above. Parallel nitrocellulose filters were incubated with radioactive oligo(dT) primed, single-stranded cDNAs obtained from total

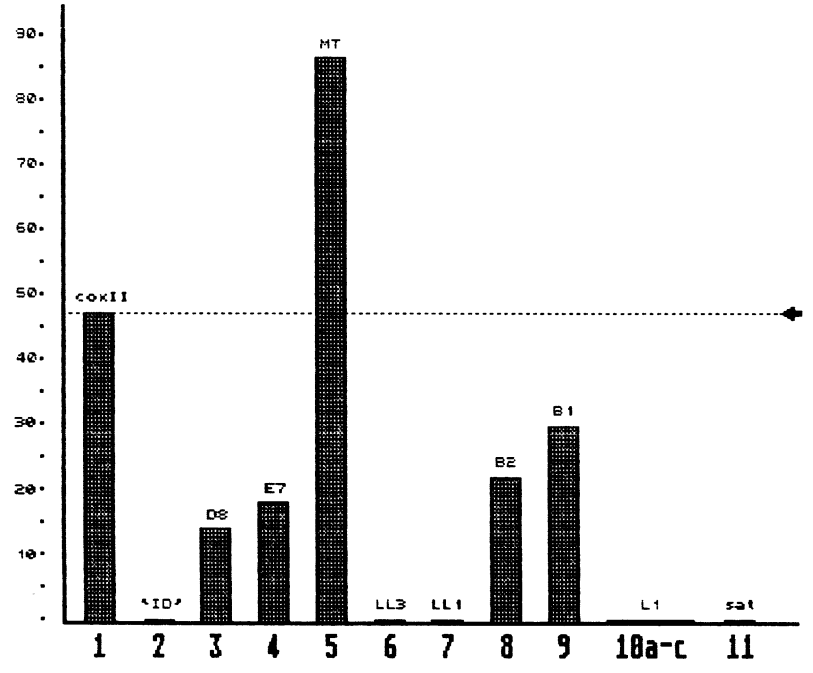

Fig. 7. Hybridization of single-stranded ^{32}P-cDNAs prepared from cytoplasmic poly(A)$^+$RNAs of total brain (TB) to immobilized clones carrying repetitive sequences. Numbering of clones is as in Fig. 6.

brain (TB), cerebral cortex (Cx), cerebellum (Cb), and kidney (K). For comparison the densitometric results of different tissues were corrected with respect to cytochrome oxidase II expression which showed a variation of about 10% between the tissues under investigation (presumably due to different exposure times).

MT sequences bound nearly twice as much radioactivity from total brain cDNAs as did cytochrome oxidase II clone CebA-D4 (Fig. 6), whereas much less radioactive cDNAs prepared from cerebral cortex and cerebellum, and even less from kidney, hybridized with the MT probe. A comparison with other repetitive sequences indicates that MT sequences are by far the most abundant reiterated DNA elements in total brain cytoplasmic poly(A)$^+$RNAs (Fig. 7). No bound radioactivity was observed for satellite DNA (lane 11), L1 sequences (lane 10), LLRep1 and LLRep3 (lanes 6 and 7), and R.dre.1 ("ID") (lane 2). B1 and B2 yielded a signal about half as intense as obtained for cytochrome oxidase II, whereas CebA-D8 and CebN-E7 yielded about one third (see Fig. 7).

DISCUSSION

In this report we describe the partial nucleotide sequence of a previously unknown interspersed middle-repetitive DNA family present in mouse and rat genomes. In contrast to other characterized LINE and SINE (1) families such as LINE-1 (3,4), AluI (7), L1Md (8,9), B1 and B2 (10,11) the initial clone carrying the repetitive sequence was isolated by random characterization of cDNA clones from a pcD-cDNA-library prepared from murine cerebellar poly(A)$^+$RNA. This different experimental approach may be responsible for the detection of an additional, and currently unknown, middle-repetitive DNA family. The entire length of this repetitive element is still unknown, but from the sequence data it is at least 370 bp long. This was deduced from the longest homologous stretch within the genomic MT clones, i.e. the homology between MT-4 and MT-28 (see Fig. 3). The 3'-border of the repetitive element remains to be determined more precisely, but might map some tens of bp around the MT-4-HincII site. As a hint for this one may take the longest homology between MT-4 and mouse Ins (19) which extends only 25 bp downstream of the MT-28-PvuII site (see Fig. 3). However, a B2 element is present in mouse Ins, which starts immediately after the MT stretch, and thus might have interrupted the complete MT sequence by retroposition.

So far there is no evidence for an A/T-rich region (as a consequence of a retroposition event) at one end of the consensus sequence as described for other repetitive elements (2,8,10,16). We expect such an A/T-tail, if at all, at the 3' end (relative to the orientation in CebA-847), since the relative 5' end has been determined on the basis of four clones (CebA-847, MT-28, MT-13Hae, and mouse Ins, see Fig. 3). However, the homologous rat sequence (rncyc450) is also truncated at this end (Fig. 3 and 4).

Thus, no reasonable speculation on the modes of amplification and integration can be made. It is also still unclear whether the positioning of MT sequences within cDNA clone CebA-847 was accomplished by use of structures concerning the 11 bp inverted repeat 5'-AACCCTTTCTT-3' (see Fig. 3). This repeat is present within the consensus sequence as well as in a portion of CebA-847 which is not homologous to other genomic MT clones. Both repeats are parted by 250 nucleotides and, as illustrated in Fig. 4, the consensus sequence region bearing the repeat is strictly conserved within the genomic clones sequenced presently. A current investigation of other MT-carrying cDNA clones will hopefully shed a light on the possible involvement of such repeats in the integration of MT sequences.

Although it is too early to postulate a special mechanism of integration

of MT sequences into brain transcription units, our hybridization data indicate (see Fig. 7) that this repetitive family is the most abundant reiterated DNA element present on cytoplasmic poly(A)$^+$RNAs from total brain when compared with characterized repetitive control clones. Even when recognizing that repetitive DNAs are more abundant on cytoplasmic poly(A)$^+$RNAs of the brain stem than of other tissues (37), the difference between genomic copy number and extent of radioactive cDNA hybridization is very pronounced (compare Figs. 5 and 7). This high abundance of MT sequences is not due to small RNAs from individual repeats, since prominent bands in the low molecular weight range could not be detected by Northern blot analyses (these blots will be published elsewhere in connection with the cerebellum-specific expression data of CebA-847).

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