Cloning of metallothionein cDNA from neonatal rat liver

J. F. B. MERCER* and P. HUDSON**
*Birth Defects Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria, Australia 3052; and **Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria, Australia 3052

(Received 13 August 1982)

A metallothionein cDNA clone was isolated from a cDNA bank prepared from neonatal rat liver poly(A)-containing RNA by a colony screening procedure using [32P]cDNA probes prepared from mRNA of either metal-induced or uninduced rat livers. Nucleotide sequence analysis of this clone showed that it contained the entire 3' untranslated region and 30% of the coding sequence for a rat metallothionein. The sequence is remarkably homologous with the mouse metallothionein-I gene.

Metallothioneins (MTs) are unusual low-molecular-weight proteins which have a high cysteine content (33%) and an absence of leucine and aromatic amino acids. Most vertebrate tissues contain two forms of MT (MT1 and MT2) that differ in amino acid sequence. MTs bind various heavy metals and are involved in zinc and copper metabolism and heavy-metal detoxification (Kågi & Nordberg, 1979). Wong and Klaasen (1979) reported that the livers of newborn rats have a transiently high level of MT, and Ketcheson et al. (1969) showed that Cu and Zn levels are also elevated in this age group. Foetal livers of cattle, sheep, and humans also have high Cu and Zn contents (Bremner et al., 1977; Widdowson et al., 1972), and it is likely that the Cu and Zn are associated with MT (Riordan & Richards, 1980). The reason for these high metal levels in the livers of foetal and neonatal animals is not clear but the molecular mechanisms of regulation are of interest and a study of these will hopefully elucidate more clearly the role of MTs in Cu and Zn metabolism in general. To further our study of the regulation of MT gene expression during development of the rat we report here the first isolation of an MT cDNA clone from neonatal rat livers and compare the sequence with the mouse MT1 cDNA sequence (Durnam et al., 1980).

Materials and Methods

Preparation of RNA

Copper-induced adult livers were obtained from male Sprague-Dawley rats 5 h after injection of CuCl\(_2\) (3 mg of metal/kg) in 0.9% saline, and neonatal livers from 1-d-old animals. RNA was prepared from the livers as described previously (Mercer et al., 1981).
Preparation of the cDNA clone bank

Double-stranded cDNA (ds cDNA) was prepared from neonatal rat liver poly(A)-containing RNA using reverse transcriptase for the initial cDNA synthesis (Wickens et al., 1978) and the Klenow fragment of DNA polymerase I for the second strand synthesis (Jacobson et al., 1974). After S1 nuclease treatment (Vogt, 1973) the ds cDNA was tailed with deoxycytidine residues using terminal transferase (Roychoudhury et al., 1976) and annealed with the bacterial plasmid pBR322 which had been cleaved with PstI and G-tailed (Chang et al., 1978). The recombinant plasmids thus formed were used to transform E. coli RR1 (Morrison, 1979). Tetracycline-resistant clones (10^4 colonies/μg plasmid DNA) were stored in 30% glycerol (in nutrient broth) at -20°C as the neonatal liver cDNA clone bank. About 70% of the colonies were ampicillin-sensitive, indicating that they contained a cDNA insert in the PstI site of pBR322.

Preparation of cDNA probes and colony screening

Poly(A)-containing RNA (200 μg/11 ml gradient) from copper-induced or normal livers was sedimented through a 5-30% sucrose gradient containing 10 mM Tris/HCl, 10 mM NaCl, pH 7.4, for 18 h at 35 000 r.p.m. using an IEC SB-283 rotor in an IEC B-60 ultracentrifuge. RNA was disaggregated by heating for 5 min at 60°C prior to centrifugation. Fractions (0.5 ml) were collected and assayed by translation in vitro in a wheat-germ system using [35S]cysteine or [3H]leucine (Mercer et al., 1981). The MT-mRNA fractions (95) were pooled and used for preparation of the cDNA probes; the >95 RNA was obtained by pooling all fractions containing higher-molecular-weight mRNA than MT mRNA. 32P-labelled cDNA probes were prepared from the 95 induced, 95 uninduced, and >95 poly(A)-containing RNA (1-2 μg) using reverse transcriptase as described by Wickens et al. (1978) except that the labelled nucleotide concentration was only 1 μM, 400 Ci/mmol. Colony filter hybridizations were carried out as described by Grunstein and Wallis (1979).

Fragment preparation and DNA sequencing

The plasmid was isolated from the selected clone (Tanaka & Weisblum, 1975) and cleaved with PstI to release the insert, which was purified by electrophoresis on an 8% polyacrylamide gel (McDonnell et al., 1979). For a colony-screening probe, the PstI fragment was digested with HpaII (Boehringer) and the shorter sub-fragment (shown by sequence analysis to be the 5' end) was isolated for synthesis of a 32P-labelled probe by the random primer method (Taylor et al., 1976).

For sequencing, the PstI fragment was cut either with HpaII or HinfI and the fragments end-labelled with [α-32P]dCTP or [α-32P]dATP respectively (Goodman, 1980). These end-labelled fragments were purified using polyacrylamide-gel electrophoresis and subjected to DNA sequence analysis by the method of Maxam and Gilbert (1980).
Fig. 1. Translation products of RNA fractions used to prepare cDNA probes. RNA fractions were translated in a wheat-germ in vitro system using $[^{35}S]$cysteine as described previously (Mercer et al., 1981). The products were blocked by carboxyamidomethylation and analysed by 17.5% polyacrylamide/sodium dodecyl sulphate gel electrophoresis. Labelled products were detected by fluorography (Bonner & Laskey, 1974). Lane 1, 9S RNA from Cu-injected rats; lane 2, 9S RNA from uninduced rats; lane 3, >9S RNA; lane 4, -RNA. The arrow indicates the position of MT determined using purified MT (Mercer et al., 1981).

Results

Identification of a metallothionein cDNA clone

Fig. 1 shows an SDS/polyacrylamide gel of $[^{35}S]$cysteine-labelled wheat-germ translation products of the 9S RNA fractions from a sucrose gradient (see 'Materials and Methods'). A prominent band with an apparent mol.wt. of 14 000 is produced with the 9S induced RNA, and this has been shown to be MT (Mercer et al., 1981). Comparison of the induced (lane 1) and uninduced (lane 2) translation products shows that the level of translatable MT mRNA is much greater in the induced fraction and that very little metallothionein mRNA is detectable in the >9S fraction (lane 3). The induction of MT mRNA by copper chloride has been estimated to be 4.5- to 9-fold (Mercer et al., 1981).
Fig. 2. In situ filter hybridization of selected clones. Some of the clones selected from the bank were plated in duplicate onto 3 separate Millipore filters and hybridized with $^{32}$P-labelled cDNA probes from 9S induced RNA (A), 9S uninduced RNA (B), and >9S RNA (C). The filters were processed as described by Grunstein and Wallis (1979) and the positive clones detected by autoradiography.

Although MT is the predominant cysteine-labelled product produced by translation of the induced RNA, this is somewhat misleading since MTs contain such a high proportion of cysteine. We estimate, however, that this mRNA fraction comprises about 10% MT mRNA (based on cell-free translation data, unpublished results), so a cDNA probe prepared from the induced RNA was expected to give a strong signal with any MT clone. Accordingly, we screened approximately one thousand colonies from our neonatal clone bank by the method of Grunstein and Wallis (1979). Some of the strongest-hybridizing clones were selected and replated together with some weakly hybridizing clones as negative controls in three replica grids on nitrocellulose and screened with $[^{32}$P]cDNA probes prepared from the 9S induced, 9S uninduced, and >9S RNA. Fig. 2 shows the result of part of this screen in which one neonatal clone, indicated with the arrows (duplicate colonies), responded in the manner expected for an MT clone; i.e. strong hybridization with the probe from induced 9S RNA (A), weaker with the probe from uninduced 9S RNA (B), and very weak with the probe from >9S RNA (C). The >9S RNA screen was included to eliminate possible confusion with fragments from larger copper-inducible RNAs which may sediment at 9S. The indicated clone termed pMT-N1 was isolated and characterized.
Characterization of pMT-N1 and other metallothionein cDNA clones

PstI digestion of the plasmid isolated from pMT-N1 released a 360-base-pair fragment which was found to be cleaved by Hpall and Hinfl but not by Tagl. The DNA sequence of the insert was obtained using the method of Maxam and Gilbert (1980). The sequence is shown in Fig. 3 and is compared with the equivalent sequence of mouse MT1 cDNA as reported by Durnam et al. (1980). The remarkable sequence homology between the rat and mouse sequences in both the coding and 3' untranslated regions confirms that we isolated a metallothionein cDNA clone, containing all the 3' untranslated region and part of the coding region. The coding region similarity to mouse MT1 indicates that our clone is probably metallothionein 1 (MT1), since the amino-acid sequence predicted from the coding sequence agrees with that found in mouse MT1 in 4 out of 5 positions where mouse MT1 and MT2 differ in sequence. The exception is the serine at position 54 in the protein (underlined), which is alanine in mouse MT1. Since the complete amino-acid sequences of rat MT1 and MT2 have not been reported, we cannot say whether the rat and mouse proteins differ in this position.

We rescreened the neonatal clone bank with a labelled probe prepared from the 5' HpaII fragment of the MT cDNA clone, to select for the longest MT cDNA clones. The insert sizes of these (18) were all found to be smaller than pMT-N1, ranging from 190 to 310 base pairs. The sequence of one of the longest neonatal clones from this rescreen was found to be identical to pMT-N1 insert except for a shorter poly(A) segment and, perhaps significantly, the 5' end of the clone terminated one base from the end of pMT-N1.

Discussion

Our screening strategy using cDNA prepared from copper-induced and uninduced RNA was successful in identifying a metallothionein cDNA clone and the amino-acid sequence predicted from the coding region suggests the clone is derived from MT1 mRNA. The screening method could therefore be used to look for other Cu-inducible proteins including MT2.

None of the MT clones identified by rescreening with the MT cDNA contained the entire MT coding region. We do not think that the absence of full-length clones is due to inefficient enzyme reactions during generation of the ds cDNA. For example, we have shown that the clone bank contains cDNA inserts ranging from 150 to >1500 base pairs (unpublished data), indicating that we have generated long inserts from other messenger RNAs. Furthermore, sequence analysis of another MT clone showed that it terminated within one base of the 5' end of the insert in pMT-N1 and many of the clones contained similar but not identical-length 5' HpaII fragments indicating they may have terminated at similar points, whereas the 3' HpaII fragments were of variable length, presumably due to different poly(A) sizes (unpublished data). We suggest, therefore, that some property of the MT mRNA is responsible for the absence of full-length clones. The two main possibilities are: secondary structure of the mRNA which prevents complete reverse transcription; or some sequence homology
Fig. 3. DNA sequence of insert from pMT-N1 compared with corresponding DNA sequence of mouse MT1. The asterisks indicate the positions of homology between the mouse (Durnam et al., 1980) and rat MT nucleotide sequences. The restriction-enzyme sites are indicated by horizontal bars, either above for the mouse cDNA sequence or below for the rat cDNA sequence.

**Hha I, Taq I, Sst II**

<table>
<thead>
<tr>
<th>MOUSE</th>
<th>AA TGT GCC CAG GGC TGT GTC TCG AAA GGC GCC GCG GAC AAG TGC ACG TGC TGT GCC TGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT</td>
<td>AA TGT GCC CAG GGC TGT GTC TCG AAA GGT GCC TCG GAC AAG TGC ACG TGC TGT GCC TGA</td>
</tr>
</tbody>
</table>

Cys Ala Glu Gly Cys Val Cys Lys Ala Ser Asp Lys Cys Thr Cys Cys Ala STOP

**Hpa II, Ava II**

<table>
<thead>
<tr>
<th>MOUSE</th>
<th>TGTGACGAAACAGCGGCTGC CACCCGTCCTTAAATTGATCGGC ACCAACCACGCTTTCTGATCGTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT</td>
<td>AGTGACGAAACGATGCTGTCCCTGCTCACTGGTAAATTCAATTGCGAACCACGCTCTTCTGCC</td>
</tr>
</tbody>
</table>

**Hinf I**

<table>
<thead>
<tr>
<th>MOUSE</th>
<th>CACCCGTTTTACTAATACGGGTCTCTAGCTAAATAAAAGCTGTCTT(A)20</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT</td>
<td>CACCCGTTTTACTAATACGGGTCTCTAGCTAAATAAAAGCTGTCTT</td>
</tr>
</tbody>
</table>

**Mse I**

<table>
<thead>
<tr>
<th>MOUSE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT</td>
<td></td>
</tr>
</tbody>
</table>
which resulted in the formation of a large hairpin loop at the 5' end that was removed by S1 nuclease. The cDNA clones do not, of course, need to be full-length to be useful as hybridization probes.

The sequence data we have presented here are sufficient to allow an interesting comparison with the mouse MT1 sequence. The degree of conservation in the coding region is very high (96.5%) and even the 3' untranslated region is highly homologous (81%). The DNA sequences of rat and mouse amylase genes show a similar degree of homology in which the coding regions are more similar than the short 3' non-coding segment (Hägenbuckle et al., 1980; MacDonald et al., 1980). The similarity in DNA sequence between the rat and mouse genes probably reflects the recent evolutionary divergence of the two species (Simpson, 1959). Recently the DNA sequence of a human MT2 cDNA has been reported (Karin & Richards, 1982) showing that the coding region is well conserved (81%) between human MT2 and mouse MT1, but the 3' non-coding sequences show little homology. It will be of interest to compare the coding and non-coding DNA sequences of metallothioneins from other species since the metal-binding sites have been highly conserved (Kägi & Nordberg, 1979).

Acknowledgements

We thank A. Grimes and I. Lazdins for their excellent assistance and J. Camakaris and D. M. Danks for discussions during this work and criticism of the manuscript. AMV reverse transcriptase was provided by Dr. J. Beard through the Office of Program Sources and Logistics, Viral Cancer Program, NCI.

References