Structure of the Gene for CAD, the Multifunctional Protein That Initiates UMP Synthesis in Syrian Hamster Cells

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Two adjacent fragments of genomic DNA spanning the gene for CAD, which encodes the first three enzymes of UMP biosynthesis, were cloned from a mutant Syrian hamster cell line containing multiple copies of this gene. The mutant was selected for resistance to *N*-(phosphonacetyl)-L-aspartate, a potent and specific inhibitor of aspartate transcarbamylase, the second enzyme in the pathway. The sizes and positions of about 37 intervening sequences within the 25-kilobase CAD gene were mapped by electron microscopy, and the locations of the 5' and 3' ends of the 7.9-kilobase CAD mRNA were established by electron microscopy and by other hybridization methods. The coding sequences are small (100 to 400 bases), as are most of the intervening sequences (50 to 300 bases). However, there are also several large intervening sequences of up to 5,000 bases each. Two small cytoplasmic polyadenylated RNAs are transcribed from a region just beyond the 5' end of the CAD gene, and their abundance reflects the degree of gene amplification.

Recent developments in molecular biology have allowed the structures of many mammalian genes to be studied at the molecular level. However, most of these genes code for specific products of terminally differentiated cells, such as globins, immunoglobulins, and albumins, and our current knowledge of the structures of genes expressed by most tissues throughout development is based on very few examples. We now present an initial characterization of the Syrian hamster gene which encodes the multifunctional enzyme that catalyzes the first three steps of de novo pyrimidine nucleotide biosynthesis, reactions that are carried out continually in most cells. This protein, called CAD after the names of its three activities (carbamyl-phosphate [P] synthetase, aspartate transcarbamylase, and dihydro-orotase), has been purified from mutant cells resistant to high levels of *N*-(phosphonacetyl)-L-aspartate (PALA), which inhibits aspartate transcarbamylase (4, 10). All the mutant cells tested have more copies of the CAD gene than do the wild-type cells (19), and mutants can be selected sequentially with higher and higher levels of PALA until they contain as many as 200 to 300 copies of this gene. Amplification of the CAD gene is maintained at or near these high levels in cells cultured continuously for as long as 10 months in the absence of PALA.

In previous work (13, 19) the 7.9-kilobase (kb) CAD mRNA was isolated and cDNA clones were prepared. Comparison of the restriction maps of CAD cDNA clones with those of homologous genomic DNA fragments indicated that many intervening sequences were present. We now describe the cloning and detailed structure of the CAD gene from a PALA-resistant Syrian hamster cell line. Several members of a family of dispersed, highly repeated DNA sequences, homologous to many polyadenylated [poly(A)⁺] cytoplasmic RNAs, are present within and near the CAD gene. In the following paper (14) we describe the homology between these repeated sequences and cytoplasmic RNAs and the presence in these repeated sequences of RNA polymerase III transcription units active in vitro. In a third paper (21) we show the chromosomal locations of CAD genes, both in cell lines with amplifications and in wild-type cells, by means of in situ hybridization to fixed metaphase chromosomes.

MATERIALS AND METHODS

Cells and cell culture. PALA-resistant (PALA^r) cell lines were selected and maintained as described by Kempe et al. (10). The PALA^r lines 97-3 and 165-28, derived from PALA^s SV28 cells (10), and the PALA^r line B5-3, derived from PALA^s BHK 21/13 cells (19), have already been described. The B8-4 line, cloned from B5-3 cells selected in 10 mM PALA, overproduces CAD by 100-fold relative to BHK 21/13 cells.

Recombinant DNA techniques. Total RNA, poly(A)⁺ cytoplasmic RNA, and cellular DNA were isolated from PALA^s and PALA^r cells as described previously

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(19). Plasmid DNA was isolated from *Escherichia coli* as described (19) except that the cells were lysed by adding 2 volumes of 0.1% Triton X-100–60 mM EDTA–50 mM Tris-hydrochloride (pH 8.1) on ice for 10 min. Lambda phage were grown and DNA was isolated as described by Thomas and Davis (18). Charon 4A and λ -100 phage were grown in *E. coli* strain LE392 or DP50 *supF* (2). λ -200 and λ gt5-lac5 (6) phage were grown in a *recA* derivative of *E. coli* C600. The derivative was produced by P1 transduction from a donor strain (obtained from K. McEntee) carrying the *recA57* allele linked to Tn10 (12; K. McEntee, personal communication) and selected for resistance to tetracycline. λ c1857 was a gift from J. Widom. A 19-kb genomic DNA fragment from the PALA^r cell line B8-4 was cloned by digesting DNA from B8-4 with *EcoRI* and ligating it to Charon 4A DNA, also digested with *EcoRI*. The DNA was packaged *in vitro* (7), and the phage were plated onto *E. coli* DP50 *supF*. The plaques were screened according to Benton and Davis (1), using pCAD₄₁ DNA (19) labeled by nick translation (16) as probe.

Gel electrophoresis and transfers to paper. DNA fragments and glyoxal-treated RNAs were separated by electrophoresis in agarose gels, stained with ethidium bromide, and transferred to diazobenzoyloxymethyl-paper as described (19, 20). ³²P-labeled DNA probes were prepared by nick translation (16), DNA or RNA immobilized on diazobenzoyloxymethyl-paper was revealed with the probes in the presence of 10% dextran sulfate (20), and the paper was washed and autoradiographed as described previously (19).

Preparation of RNA-DNA hybrids and electron microscopy. Amounts of phage Charon 4A and λ -100, or λ c1857 and λ -200, equivalent to 0.1 μ g of DNA each were combined in 50 μ l of 80% formamide (recrystallized), 0.4 M NaCl, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer (pH 6.8), 1 mM EDTA, and 10 ng of poly(A)⁺ RNA from 16S-28S cells, previously enriched for the relatively large CAD mRNA by sedimentation in a sucrose gradient. The mixture was heated at 68°C for 2 min and at 51.5°C for 3 h, then cooled to 25°C. Samples of 10 μ l taken after 30 and 60 min at 25°C were diluted to 100 μ l with 40% formamide–100 mM Tris-hydrochloride (pH 8.5)–10 mM EDTA–50- μ g/ml cytochrome *c*, spread over a hypophase of 10% formamide, 10 mM Tris-hydrochloride (pH 8.5), and 1 mM EDTA, picked up on Parlodion-coated grids, and rotary shadowed (8). The grids were examined in a Philips EM300 microscope, hybrid structures were photographed, and the images were measured using a Hewlett-Packard 9810A calculator and 9864A digitizer.

RESULTS

Recombinant phage carrying fragments of the CAD gene. Previous work had shown that a 2.3-kb cDNA clone, pCAD₄₁, is complementary to the 3' portion of the 7.9-kb CAD mRNA and that it hybridizes to a single 19-kb *EcoRI* fragment in DNA from both PALA^r and PALA^s cells (19). A library of *EcoRI* fragments from PALA^r cell line B8-4 was cloned in phage Charon 4A and screened with labeled DNA from pCAD₄₁. Six plaques out of 6 $\times$ 10⁴ were positive. Analysis of

the DNA from four of these showed that they all contained the same 19-kb *EcoRI* fragment of genomic DNA. Nick-translated probe from one of these phage (λ -100) hybridized to a 19-kb *EcoRI* fragment present in much greater amount in PALA^r cells than in PALA^s cells and also hybridized to a 7.9-kb poly(A)⁺ RNA which was much more abundant in PALA^r cells than in PALA^s cells (data not shown).

The restriction map of the 19-kb *EcoRI* fragment in λ -100 is shown in Fig. 1. The direction of transcription of the CAD gene was determined by comparison with the known transcriptional polarity of pCAD₄₁ (19). Initial experiments showed that λ -100 did not contain all the sequences in the 7.9-kb CAD mRNA. Therefore, *Bam*HI-*Eco*RI fragment 104, derived from the 5' end of λ -100, was cloned into pBR322 (3), and the recombinant, designated p104, was used to screen a phage bank made from B8-4 DNA cut with *Sst*I and cloned into λ gt5-lac5. Phage positive in the screening contained a 13-kb insert, in agreement with the size expected from Southern blot (17) analysis of the genomic DNA using p104 as probe (not shown). The restriction map of this new phage (λ -200) is also shown in Fig. 1. All of the fragments shown in Fig. 1, except fragment 103, have been subcloned into pBR322.

All of the cloned genomic DNA fragments that contain portions of the CAD gene hybridized to fragments of identical size in blots of DNA from PALA^s or PALA^r cells (data not shown). It was shown previously that the CAD protein (4) and mRNA (13) obtained from overproducing PALA^r mutants are not distinguished functionally or structurally from the protein or mRNA of PALA^s wild-type cells. These results indicate that the CAD genes in the mutant or wild-type cells are very similar or identical.

Intervening sequences in the CAD gene. Previous work (19) had shown that the CAD gene contains many intervening sequences. We determined their sizes and locations by examination in the electron microscope of three-stranded structures formed between CAD mRNA, phage containing the 19-kb or 13-kb genomic fragments, and the parent λ phage without an insert of hamster DNA (Fig. 2). These structures have double-stranded DNA at each end, corresponding to shared sequences from the left and right arms of λ , and an internal portion in which the mRNA is hybridized to one of the single strands. The lengths of the double-stranded λ arms and the unhybridized single strand are known and serve as size standards for measurements within the RNA-DNA hybrid region. Since the lengths of the λ arms are different, the polarity of the RNA-DNA hybrid can also be defined. Figure 2a shows the structure formed with CAD mRNA and a heteroduplex between DNAs from λ -100

FIG. 1. Restriction map of the CAD gene. The top line shows the location of restriction sites within the cloned region. The symbols are: R, *EcoRI*; B, *BamHI*; S, *SstI*. The heavy lines indicate the genomic DNA contained in the two phage, and the lighter lines beneath them indicate fragments cloned into pBR322 (with the exception of fragment 103).

and Charon 4A. Figure 2b shows the structure formed with CAD mRNA and a heteroduplex between DNAs from λ -200 and λ c1857. The arrows indicate the ends of the RNA-DNA hybrid region.

Maps of six hybrid structures from each of the two phage-containing inserts of hamster DNA are shown in Fig. 3, with the 5' ends toward the left. The maps are aligned so that one end of an easily recognized intervening sequence coincides in each case. A 1.75-kb deletion in the largest intervening sequence of λ -100, represented as a dashed line in Fig. 3, often occurs during cloning. The deletion removes DNA only from within the intervening sequence. Several pieces of evidence, as follows, indicate that the bumps evident in the RNA-DNA hybrid regions of Fig. 2 represent intervening sequences too small to be seen as loops: the bumps occur in similar positions in many independent examples, they are not seen in regions of double-stranded DNA within the same structures, and they are often associated with sharp bends in the RNA-DNA hybrid regions (double-stranded DNA in the same structures is generally smooth). Furthermore, the measured lengths of the hamster DNA inserts are much smaller than the sizes of the genomic *EcoRI* fragments if the bumps are not included. Even when all of the visible intervening sequences are included in the measurements, there remains a deficit of about 1.5 kb in λ -100,

possibly because the sizes of the small intervening sequences (assumed to be about 50 bases) are underestimated and possibly also because very small intervening sequences might have been missed. About 37 intervening sequences are seen reproducibly, as represented in Fig. 3.

Genomic location of the 3' end of CAD mRNA. A cDNA clone of CAD mRNA (pCAD₁₃) has been shown by sequence analysis (Shigesada and Stark, unpublished data) to contain a run of 45 A residues at the 3' end of the cDNA insert, followed directly by the run of C residues introduced by the cloning method used (19). Since the cDNA clones were produced by elongation with reverse transcriptase of an oligodeoxythymidylic acid primer hybridized to the poly(A) tract on the RNA, the sequence evidence indicates that the 3' end of CAD mRNA is within pCAD₁₃. To show that this region is within λ -100, blots of B8-4 DNA digested with *EcoRI* or *BamHI* were probed with nick-translated pCAD₁₃ DNA. Figure 4 shows that pCAD₁₃ labels only the 19-kb *EcoRI* fragment corresponding to λ -100 and the 8.6-kb *BamHI* fragment 102. No other bands are seen on longer exposure.

Genomic location of the 5' end of CAD mRNA. Since none of our cDNA clones contains the 5' end of the 7.9-kb CAD mRNA, we used both RNA-DNA heteroduplex mapping and probing of RNA blots with genomic DNA fragments to

FIG. 3. Map of intervening sequences in the CAD gene. The thick solid lines represent the coding sequences of the gene, the thin lines represent intervening and flanking sequences, the open bars represent double-stranded regions in the phage arms, and the triple lines represent the regions of single-stranded phage DNA.

determine its location. As shown in Fig. 3, the 5' end of the RNA appears to be about 5.5 kb from the left end of λ -200. To test the possibility that the 5' end might be outside λ -200, we hybridized CAD mRNA to a cosmid containing a complete functional CAD gene (16a). The cosmid contains 45 kb of hamster DNA and includes all of both λ -100 and λ -200, plus at least 10 kb of genomic DNA beyond the 5' end of λ -200. It is capable of transforming to prototrophy mutant Chinese hamster ovary cells auxotrophic for uridine and deficient in CAD (15). Figure 5 shows a heteroduplex between this cosmid and CAD mRNA. The 5' end of the RNA hybridizes to the same region as in λ -200, suggesting that there is no substantial region of homology to CAD mRNA for at least 10 kb beyond the end of λ -200. However, if a very small region of homology were present, a loop might not be stable enough to be observed in the electron microscope.

Hybridization of cloned DNA fragments from the CAD gene to blots of poly(A)⁺ RNA from PALA^r and PALA^s cells gives results consistent with the locations of intervening sequences and the ends of the RNA, as defined by other methods. Figure 6 shows that when cloned fragments of λ -200 are used as probes of poly(A)⁺

RNA from PALA^r 165-28 cells, only plasmids p201, p202, and p206 hybridize to the 7.9-kb and 10.2-kb CAD RNAs, as predicted by the previous data (the 10.2-kb RNA is apparent on longer exposure). Plasmid p205 appears to contain only intervening sequence DNA. The results of the heteroduplex mapping described above place the 5' end of the RNA somewhere within fragment 206. From a combination of the results above, we estimate that the CAD gene spans about 25 kb of genomic DNA, with about 8 kb of coding sequences and 17 kb of intervening sequences.

Other transcriptional units within λ -200. Figure 7A shows the hybridization of subclones p203, p207, and p208 to cytoplasmic poly(A)⁺ RNA from 165-28 cells. Plasmids p207 and p208, which are beyond the 5' end of CAD mRNA according to the evidence from electron microscopy, hybridize to discrete RNAs of approximately 1.0 and 0.8 kb, respectively. p203 contains DNA complementary to both 7.9-kb CAD mRNA and the smaller RNAs. To confirm that the transcripts are produced from the region just beyond the 5' end of the CAD gene, total poly(A)⁺ RNA from wild-type SV28 cells was also probed with nick-translated DNA from sub-

FIG. 2. Electron micrographs of three-stranded structures formed between CAD mRNA and λ phage with and without an insert of hamster DNA. (a) Structure formed between CAD mRNA, λ -100, and Charon 4A. (b) Structure formed between CAD mRNA, λ -200, and λ c1857.

FIG. 4. Hybridization of ^{32}P -labeled pCAD₁₃ DNA to a blot of B8-4 DNA digested with *Eco*RI or *Bam*HI.

clones p203, p207, and p208 (Fig. 7B). When the extents of hybridization with the p203 probe are compared, it is clear that the two smaller RNAs are overproduced to a degree similar to that of CAD mRNA in the mutant cells, indicating that they are very likely to be transcribed from amplified DNA. The background smears and hybridization to unresolved small RNAs evident in Fig. 7 reflect the presence of members of a dispersed, highly repeated family of DNA sequences within the genomic fragments used as probes. Characterization of these repeated sequences is presented in the following paper (14).

Partial characterization of 10.2-kb CAD RNA.

As shown above, the 3' end of the 7.9-kb CAD mRNA is about 1.5 kb from one end of λ -100. A 10.2-kb CAD RNA, observed previously in some PALA^r cell lines (19), hybridizes to all genomic DNA fragments that have homology to the 7.9-kb mRNA. To determine the origin of the extra sequences in the 10.2-kb CAD RNA, poly(A)⁺ RNA was isolated from PALA^r cell

FIG. 5. Heteroduplex formed between CAD mRNA and a cosmid containing the entire CAD gene. The cosmid DNA was nicked by mild DNase I digestion, and heteroduplexes with CAD mRNA were formed as described for the phage except that the incubation at 25°C was omitted.

FIG. 6. Hybridization of ^{32}P -labeled DNA from subclones of λ -200 to blots of poly(A) $^{+}$ RNA from PALA $^{+}$ cells. Identical blots of poly(A) $^{+}$ RNA from 165-28 cells were prepared and probed with nick-translated DNAs.

lines 97-3 (which does not contain high levels of this RNA) and 165-28 (which does contain the 10.2-kb RNA). The RNA was fractionated in agarose gels and blotted to diazotized paper as described previously (19). Identical blots were then hybridized with nick-translated probes prepared from plasmids p104 or p101. Plasmids p104 and p101 contain sequences homologous to regions near the 5' end or just beyond the 3' end, respectively, of the CAD mRNA. The autoradiogram shown in Fig. 8B shows that p101 hybridizes strongly to the 10.2-kb CAD RNA and weakly to the 7.9-kb CAD mRNA, whereas p104 hybridizes strongly to both RNAs (Fig. 8A). This result indicates that at least some of the extra sequences in the 10.2-kb CAD RNA come from the region beyond the 3' end of the normal CAD messenger. We now know that 165-28 has a novel joint just beyond the 3' end of the CAD gene and that this joint is absent in several mutants that do not produce the 10.2-kb RNA (O. Brison, J. Zieg, F. Ardeshir, J. Meinkoth, G.

M. Wahl, and G. R. Stark, unpublished data). Aberrant transcription or processing in the region of this joint might well account for the 10.2-kb RNA. The small amount of hybridization to 7.9-kb mRNA seen with the p101 probe in this experiment is not in agreement with the location determined above for the 3' end of the 7.9-kb mRNA. Both this hybridization and the diffuse smears of hybridization evident in lane 1 of Fig. 8B, for example, are probably due to the presence within p101 of repeated DNA sequences (14).

DISCUSSION

The CAD gene from Syrian hamsters contains many intervening sequences, comparable to the number in the vitellogenin gene of *Xenopus laevis*, which codes for a protein of comparable size and has about twice as much intervening sequence DNA as coding DNA (22). It is striking that most of the intervening sequences in the CAD gene are quite small (50 to 300 bases) and that all but one of the coding sequences are also small (100 to 400 bases). The meaning of this regular periodicity and of the positions of the few very large intervening sequences in terms of the evolution of the gene or the functional significance of the intervening sequences is unclear. However, the suggestion of Gilbert (9) that intervening sequences may be the sites of recombinational events which brought together ancestral elements or domains of proteins can be tested in the future with the CAD gene. The three enzymatic domains contained within the multifunctional CAD protein were probably brought together long after they were formed. Prokaryotes have three unlinked genes for the three enzymes, whereas in *Neurospora* (23) carbamyl-P synthetase and aspartate transcarbamylase are contained in a single polypeptide, without dihydro-orotase. In *Drosophila* (11) and in all mammalian species investigated, the three enzymes appear to be linked in a single polypeptide. Thus, the joining of primitive subdomains to produce single functional enzymes probably preceded in evolution the joining of these monofunctional enzymes into a multifunctional protein, and this latter type of joining appears to have occurred in at least two steps. Studies in progress in two other laboratories are aimed at defining the organization of the enzymatic domains in the hamster CAD protein. The three enzymatic activities can be separated from each other by partial digestion with trypsin (5) or elastase (D. R. Evans, M. I. Mally, and D. R. Grayson, Fed. Proc. 40:1830, 1981). From the sizes of the fragments that contain the three individual activities it appears that the domains for carbamyl-P synthetase, aspartate transcarbamylase, and dihydro-orotase have molecular

FIG. 7. Hybridization of ^{32}P -labeled fragments of λ -200 to blots of cytoplasmic poly(A) $^{+}$ RNA from PALA $^{+}$ and PALA $^{-}$ cells. Identical blots of poly(A) $^{+}$ RNA from PALA $^{+}$ 165-28 cells (A) or PALA $^{-}$ SV28 cells (B) were probed with nick-translated DNA. The exposure for blot (B) was much longer than for blot (A).

weights of about 125,000, 40,000, and 45,000, respectively. The most likely order of the domains is dihydro-orotase, carbamyl-P synthetase, and aspartate transcarbamylase, but it is not known which domain is at the amino terminus. The lengths of mRNA necessary to code for these domains are 3,750 bases for carbamyl-P synthetase, 1,200 bases for aspartate transcarbamylase, and 1,350 bases for dihydro-orotase.

As shown in the third paper in this series (21), the average size of the amplified unit that includes the CAD gene is about 500 kb, large enough to contain many other transcriptional units whose products might also be amplified. We demonstrate in this paper that the products

of at least two other transcriptional units are coamplified with CAD. These units encode cytoplasmic poly(A) $^{+}$ RNAs approximately 0.8 and 1.0 kb long. Recently, another cytoplasmic poly(A) $^{+}$ RNA approximately 3.8 kb long has been shown to be coamplified in PALA $^{+}$ cells. Its transcriptional unit lies further away from the 5' end of the CAD gene than the two described above (O. Brison, S. Ardeshtir, and G. R. Stark, unpublished data). Although it is not yet known whether any of these three poly(A) $^{+}$ RNAs is translated into proteins, their overproduction illustrates the possibility that genes other than the one being selected for can be coamplified and that their products can be overexpressed.

FIG. 8. Homology of fragment 101 to the 10.2-kb CAD RNA. (A) Hybridization of fragment 104 to a blot of poly(A)⁺ RNA from PALA⁺ cell line 97-3 (lane 1) or 165-28 (lane 2). (B) Hybridization of fragment 101 to a blot identical to the one used in (A).

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