We have studied the expression and regulation of the rat testis cytosolic aspartate aminotransferase gene. The cytosolic aspartate aminotransferase activity was 5-fold lower in the testis than in the liver and kidney. A 1.9-kilobase mRNA form was detected in the rat testis in contrast to the 2.1- and 1.8-kilobase forms present in other organs. Using Northern blot and S1 mapping analyses, we found that the proximal polyadenylation site was almost exclusively used in the testis as opposed to other organs where the distal site was preferentially used. RNase protection and primer extension analysis showed that transcription was initiated at multiple sites in all organs, but the pattern of those start sites was different in the testis; in particular, a novel transcription start site was specifically detected in this organ (at position -115 from the translation start site). This site was first observed in 29-day-old rats and was maximally utilized in the adult testis. DNase I footprinting using testis nuclear extracts revealed the presence of three sites of DNA-protein interaction in the 250-base pair proximal promoter, a pattern similar to the one found using liver nuclear extracts. However, the proteins bound had different properties as shown by gel retardation experiments. We conclude that the pattern of transcription initiation and the polyadenylation site selection of a housekeeping gene can be tissue-specific.

Cytosolic aspartate aminotransferase is involved in several central metabolic pathways including the malate-aspartate shuttle (1). This enzyme activity is present in all tissues and cells tested (2). The gene coding for the rat enzyme has been cloned, and the corresponding promoter has been sequenced and characterized (3). This promoter displays some properties that are typical of housekeeping gene promoters as follows: it is GC-rich, contains several putative Sp1 binding sites, and lacks a typical TATA box. The mouse promoter has the same properties (4). Like most of the other TATA-less promoters, transcription of the cAspAT gene starts at multiple sites. In some TATA-less promoters, unique transcription start sites are found. In these cases, alternative initiation elements like the "initiator" sequence determine the location of the actual transcription start site (5). The absence of a strong initiation element in some housekeeping gene promoters presumably leads to a cluster of several transcription initiation sites.

Although the cAspAT gene is ubiquitously expressed, the structure and the regulation of the cAspAT gene promoter display specific properties that are atypical of housekeeping gene promoters. The cAspAT promoter contains six CCAAT boxes and several glucocorticoid-responsive elements (3). In agreement with the presence of the latter elements, the activity of this promoter is regulated by glucocorticoids in hepatoma cells (6, 7). It is also regulated by CAMP and insulin (6, 7). Furthermore, protein-DNA interaction studies have shown that different nuclear proteins bind to this promoter in different tissues (8). These proteins are members of the C/EBP family or other CCAAT-binding proteins such as CP1 and NF1. A surprising feature about this promoter is that the different transcription start sites are differentially regulated by glucocorticoids (3). This observation suggested that these sites were under the control of different elements within the cAspAT promoter. As a consequence, the presence of several transcription start sites may not be due to a weak specificity of the transcription initiation machinery but could be stringently controlled by different regulatory mechanisms.

The expression of several genes has been studied in the testis (9, 10). Some genes, such as protamines genes, are specifically expressed in the testis and not in other organs (11). Other genes are expressed in various organs including the testis, but, in this tissue, their expression often exhibits specific characteristics. These differences include the use of alternative promoters or transcription start sites, alternative splicing, or different polyadenylation sites (9, 12, 13). We have looked for such differences in the expression of the housekeeping gene coding for cAspAT in the testis, as compared with other organs. Indeed, it was of interest to find out whether genes that are widely expressed display tissue-specific modifications in their expression and in the maturation of their mRNAs. In the present work, we demonstrate that the cAspAT gene expression in the testis is characterized by specific modifications in the polyadenylation site selection and in the transcription initiation pattern.

**MATERIALS AND METHODS**

**RNA Preparation**—Total RNA was isolated from different tissues using the guanidium thiocyanate extraction method described by Chomczynski and Sacchi (14). Briefly, 1 g of tissue was homogenized in 10 ml of guanidium thiocyanate solution (4 mM guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) using an homogenizer followed by water-saturated phenol and chloroform-isoamyl alcohol (29:1; v/v) extractions. The aqueous phase was transferred to a fresh tube, and RNA was precipitated in 2-propanol. The RNA pellet was resuspended in 500 μl of water, and RNA was precipitated in 2-propanol. The final RNA pellet was resuspended in 500 μl of H2O. Poly(A)+ RNA was prepared by subjecting total RNA to two cycles of oligo(dT)-trisacryl chromatography (IBF) as described by Aviv and Leder (15). In some experiments, liver and kidney total RNA was extracted according to Chirgwin et al. (16).

**Northern Blot Hybridization**—Poly(A)+ RNA (2–5 μg) was electrophoresed on a horizontal 1.2% agarose, 2.2 M formaldehyde gel (17) and...
transferred to a nitrocellulose membrane (Hybond N, Amersham Corp.). The membrane was prehybridized for 24 h at 42 °C in 50% formamide, 5 x SSC, 1 x Denhardt’s solution, 50 mM sodium phosphate, pH 6.5, 0.2% SDS, and 250 µg/ml denatured salmon sperm DNA and then hybridized overnight at 42 °C in the same medium containing 10% dextran sulfate and 2 x 10⁶ cpm/ml denatured cDNA probe (6). The membrane was washed in 0.1 x SSC, 0.1% SDS at 68 °C to reduce the background, and autoradiographed for 24–72 h at ~80 °C using an intensifying screen and Amersham MP films.

S1 Mapping—Plasmid pGEM 4B21 carrying the cAspAT cDNA (18) was digested by the NcoI and MscI restriction enzymes, and the small fragment (400 bp) corresponding to bases 1366-1768 was purified. MscI and Amersham MP films.

SDS, and 250 pg/ml denatured salmon sperm DNA and then hybridized a volume of 20 µl containing 80% formamide, 40 to 50 pg of total RNA at 65 °C for 10 min and then at 30 °C overnight in the presence of [α-32P]dATP (300 Ci/mmol, Amersham Corp.) and dCTP (1 mM). This reaction generated a 3' end-labeled antisense probe (19). The probe (1 x 10⁶ cpm) was denatured at 100 °C for 5 min and hybridized to 50 µg of total RNA at 65 °C for 10 min and then at 30 °C overnight in a volume of 20 µl containing 80% formamide, 40 µx Pipes, pH 6.4, 1 mM EDTA, pH 8, and 400 mM NaCl, as previously described (3).

S1 nuclease digestion was carried out at 30 °C for 60 min by the addition of 300 µl of 560 mM NaCl, 100 mM sodium acetate, pH 4.5, 2 mM ZnSO4, 6 µg of boiled salmon sperm DNA, and 400 units of S1 nuclease (Boehringer Mannheim). The reaction was carried out at 30 °C for 60 min. The reaction was stopped by addition of a solution containing 4 µM ammonium acetate, 20 mM EDTA, pH 8, and 40 µg/ml tRNA for 1 h at 37 °C and was followed by ethanol precipitation. The size of the protected fragments was analyzed on a sequencing gel.

**Primer Extension Analysis—Oligonucleotides were 5' end-labeled, using [α-32P]dATP (300 Ci/mmol, Amersham Corp.) and T4 polynucleotide kinase to a specific activity of 6 x 10⁶ cpm/ml (19). Poly(A)+ RNA (10 µg) were added to 1.6 x 10⁶ cpm of labeled oligonucleotide, and the mixture was ethanol-pellet. The pellet was resuspended in 30 µl of 160 mM Hepes pH 7.4, 1 mM EDTA, and 0.4 mM NaCl. The hybridization reaction was carried out overnight at 30 °C. The mixture was then ethanol-pellet. The pellet was resuspended in 30 µl of reverse transcription buffer containing 50 µl Tris-HCl, pH 8.3, 10 mM MgCl2, 50 mM KCl, 3 mM dithiothreitol, 0.1% Nonidet P-40, 0.45 mM dNTP, 25 units of RNasin (Promega), and 40 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Following an incubation at 42 °C for 90 min, the reaction was stopped by addition of 20 mM EDTA and then incubated with 30 µg of RNase A for 30 min at 37 °C.

Following an extraction with a phenol-chloroform-isooamyl alcohol (25:24:1; v/v/v) mixture, the extended DNA was ethanol precipitated in 2 µM ammonium acetate and analyzed on a sequencing gel. In all sequencing gels, the sizes were determined by comparison with molecular weight markers or a sequence ladder.

**Ribonuclease Mapping**—A 530-bp fragment, including part of the cAspAT gene first exon and of the cAspAT gene promoter, was isolated according to Vaulont et al. (21) with some modifications. The binding reaction was performed in a final volume of 25 µl containing 50 mM NaCl, 50 mM KCl, 1 x 10⁶ cpm EDTA, 5 mM MgCl2, 2 mM dithiothreitol, 4 mM spermidine, 15% glycerol, 100 µg/ml bovine serum albumin, 10 mM Hepes pH 8, and 250 ng of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) as carrier.

The nuclear proteins (50 µg for testis and 30 µg for liver) were preincubated for 15 min on ice with or without the competitor oligonucleotides. Then, about 1 ng of labeled probe (2 x 10⁶ cpm) was added, and the incubation was continued for 15 min on ice. After adjusting the concentration of CaCl2 to 2.5 mM and incubating for 1 min at 20 °C, DNase I was added, and the digestion was carried out at 20 °C for 1 min. Subsequent handling of the DNA was performed as described (21).

**Gel Retardation Assay—Probes were double-stranded oligonucleotides labeled using the Klenow fragment of DNA polymerase I (8).** Protein-DNA binding was performed under the conditions described for the footprinting experiments except that 1 µg of poly(dI-dC) was used as carrier and 3 µg of nuclear proteins were added to the probe. After 15 min on ice, the samples were directly loaded onto a 6% polyacrylamide gel in 0.5 x TBE (50 mM Tris-borate, pH 8.3, 1 mM EDTA). The gel (0.2 x 16 x 16 cm, thickness x width x length) was pre-electrophoresed for about 1 h at 100 V for 90 min in 0.5 x TBE buffer and electrophoresed at 300 V at 4 °C for about 2 h.

**Oligonucleotides**—The oligonucleotides used in this study had the following sequences: CT₁, 5'-ATC GCG ATG GAA TCT GAG TCA TAC AAG CCT CCG ATAAGAT-3' (-265, -226); OL₂, 5'-TCC TCT GTG ACC CCC AGG CAC-3' (-136, -106); OL₃, 5'-GGA GTC CGC AAT GAG CAG CAC AAC-3' (+46, +75); OL₄, 5'-TCC ACT GCA TTG GTC TCA TAC AAG CCT CGT ATAAGA T-3' (-265, -226); OL₅, 5'-TCC TCT TGA ATT GCC TAA TAG ACC CTT GTC CGC CC-3' (-223, -189). **Numbers in parentheses refer to the position of the oligonucleotides in the cAspAT gene promoter (3).**

**RESULTS**

Cytosolic and mitochondrial aspartate aminotransferase were assayed as described by Parli et al. (22). In the adult rat testis, cAspAT activity (0.017 units/mg of protein) is 5-fold lower than in the kidney (0.075 units/mg of protein). The Kₘ of the enzyme for aspartate is similar in these organs. The activity of the mitochondrial enzyme is 10-fold lower in the testis than in the other organs confirming the coregulation of the two isoenzymes in the different tissues (not shown).

In previous studies, Northern blot analysis of the cAspAT mRNAs revealed the presence of two mRNA species of 2.1 and 1.8 kb in length in various tissues and cells (6, 18, 23). These two forms differ in the choice of the polyadenylation site (18, 23). Fig. 1A shows that these two mRNA forms are found in the liver in three different species, human, rat, and mouse. The relative abundance of these mRNA forms differs slightly; the ratio of the abundance of the small form versus the large form...
The liver from hydrocortisone-treated animals

is higher in the human and the rat than in the mouse.

The previous experiments have shown that a tissue-specific transcription start sites, a primer extension assay was carried out using the primer CT1 (−30, +2). This assay revealed the presence of several transcription start sites in the liver and kidney (Fig. 4), in agreement with the protection assay. Furthermore, it confirmed the presence of a specific start site in the testis at position −115. No extension products corresponded to the other testis-specific bands detected in the Rnase protection assay, suggesting that they may correspond either to degradation products or to minor transcription start sites.

The previous experiments have shown that a tissue-specific transcription start site was found in a housekeeping gene promoter. In the experiment depicted in Fig. 5, we have tested the developmental regulation of this site. Testis poly(A)^+ mRNA was prepared from 15-, 21-, 29-day-old, and adult rats and were analyzed by primer extension experiments. Those ages were
of poly(A)+ RNA from rat kidney, the promoter.
hybridized to an oligonucleotide complementary to the region on a sequencing gel were performed as described under the cAspAT gene promoter (oligonucleotide CT1). The extension and the migration on a sequencing gel were performed as described under "Materials and Methods." tRNA was used as control. Sizes (in kb) are indicated by arrows. B, similar experiment using poly(A)+ RNA from testis of 11- (T11), 15- (T15), 22- (T22), 29- (T29) day-old, and adult (T) rats. The blot was hybridized with the cAspAT probe. Sizes (in kb) are indicated by arrows. B, similar experiment using poly(A)+ RNA from testis of 11- (T11), 15- (T15), 22- (T22), 29- (T29) day-old, and adult (T) rats as well as from liver (LA) and kidney (KA) of adult rats. C, same blot as in B hybridized with α-tubulin cDNA probe.
germ cells at the round spermatid stage. Northern blot analysis (Fig. 6A) of the same mRNAs revealed that the 1.9-kb testis-specific band was slightly detectable in 29-day-old rats and markedly increased in adult rats. A similar experiment shown in Fig. 6B confirmed this result and further demonstrated that the amount of the 1.9-kb cAspAT mRNA form was increased in adult testis, relative to the amount of α-tubulin mRNA (Fig. 6C). This increase was found to be of at least 5–10-fold when band intensities were quantitated by scanning densitometry. Since this cAspAT mRNA form was clearly predominant at the adult stage, it probably comprises mRNAs resulting from transcription initiation at the multiple sites including the testis-specific site. We have attempted to probe the Northern blot with an oligonucleotide (−115, −80) corresponding to the mRNA specifically expressed in the testis. However, because this region is extremely rich in GC (more than 90%), we have failed to distinguish the specific from the nonspecific hybridization. The increase in the amount of cAspAT mRNA in the testis during development was accompanied by only a slight increase in cAspAT activity in adult rats as compared with 8-day-old rats (0.021 versus 0.014 units/mg of protein, respectively).

Since the pattern of the transcription initiation sites in the testis is different from the one found in the liver, we have compared the pattern of DNA-protein interactions in the cAspAT promoter in both organs. DNase I footprinting analysis was carried out using either liver or testis nuclear extracts, and a 260-bp promoter fragment as a probe (−286, −26). The same regions were protected from DNase I digestion in both cases (Fig. 7). When a more distal probe (−684, −225) was used, no additional protection was observed in the testis extracts as compared with the liver extracts (data not shown).

The three protected regions in the (−286, −26) fragment correspond to binding sites of C/EBP-like proteins in the liver (8). Using oligonucleotides spanning these footprints, we have analyzed the binding of testis, liver, and brain nuclear proteins by electrophoretic mobility shift assay (Figs. 8 and 9). The brain nuclear proteins bind to the same sites as the liver proteins but
are heat-sensitive, as previously shown (8). In conclusion, although the same DNA sites are bound by nuclear proteins in testis, brain, and liver, the DNA protein complexes have different properties in the three organs.

**DISCUSSION**

The major conclusion from this study is that there are tissue-specific differences in the pattern of transcription and maturation of a housekeeping gene product. The expression of the cAspAT housekeeping gene was examined in several tissues including the testis. In all rat tissues and cells tested except the testis, there are two mRNA forms, a major one at 2.1 kb and a minor one at 1.8 kb generated by the use of a distal and a proximal polyadenylation site, respectively. In the testis, a specific mRNA form at 1.9 kb is observed. The 3' and 5' ends of this mRNA form have been studied, and specific properties have been found in both cases.

The testis-specific mRNA form results from the use of the proximal polyadenylation site. Thus, while the distal polyadenylation site is preferentially used in all other organs, the proximal one is almost exclusively used in the testis. There are other examples of alternative polyadenylation site selection in the testis, namely the murine β1 galactosyl transferase (27) and the protooncogene c-abl (28). In the latter case, the polyadenylation signals used in the testis do not correspond to the consensus AUAAA sequence but rather to uncommon modified sequences. However, this is apparently not a general property of mRNA maturation in the testis, since the cAspAT signal used in this organ is identical to the consensus sequence. One common feature of the polyadenylation site selection of the c-abl, β1 galactosyl transferase, and cAspAT genes is that the proximal polyadenylation sites are preferentially used in the testis yielding mRNAs with shorter 3' untranslated regions. If this observation is confirmed in other cases, it could reflect specific differences in the polyadenylation machinery in the testis. Furthermore, shorter 3'-untranslated regions could modify the stability and the translational efficiency of the corresponding mRNAs.

The pattern of transcription initiation is different in the testis as compared with other organs. As we have previously shown, there are several transcription start sites within the cAspAT gene promoter, in good agreement with the absence of a TATA box or a typical initiator element in this promoter. As expected, the pattern of transcription initiation is similar in several organs. The most striking difference, in the testis, is the presence of an upstream start site at position −115 (+1 corresponds to the A of the first methionine codon) and a decreased efficiency of the downstream sites. The window of transcription initiation in the other organs lies between position −35 and −91. These differences could contribute to a small difference in the average mRNA size between the testis-specific 1.9-kb mRNA population and the minor 1.8-kb form present in the other organs, since both forms result from the use of the same polyadenylation site. It is possible that other properties such as poly(A) length could contribute to the size difference observed by Northern blot.

Testis-specific transcription start sites have been detected in genes that are otherwise expressed in other organs. In several cases, the testis-specific start sites are located several hundred or several thousand bp away from the somatic start sites. This is the case for angiotensin-converting enzyme (29), proenkephalin (30) and cytochrome c (31) as well as for α-tubulin (32). In these cases, there are specific testicular promoters that most probably do not share regulatory elements with the corresponding somatic promoters. In the case of the angiotensin-converting enzyme gene, the testis-specific promoter has been analyzed using transgenic mice (29, 33). Sequence similarities...
have been found with the promoter of the protamine 1 gene, a germ line-specific gene. The specificity of the latter gene was also proven using transgenic mice (34). In both cases, a cAMP-responsive element-like element was suggested to be critical for expression. In the HSV thymidine kinase gene, a cryptic testicular promoter was uncovered in transgenic mice experiments (35). This promoter bears homologies with housekeeping gene promoters in that it contains no TATA box and is GC-rich. Despite the increasing amount of data on factors common to several testis-specific promoters, the molecular basis of germ line-specific expression that may include positive and negative regulators has yet to be deciphered.

The observations made here on the transcription initiation of the cAspAT gene are clearly different from those cited above. Indeed, the testis-specific start site in the case of the cAspAT gene promoter is only 25 bp upstream of the window of transcription start sites present in somatic tissues. This is similar to the observations made on the rat farnesyl pyrophosphate synthetase gene promoter, where several testis-specific transcription start sites are located 25–100 bp upstream of the somatic start sites (36). In this case, the somatic start sites are clustered into two groups that are preceded by TATA boxes. In contrast, the testis-specific start sites are spread over a region of 90 bp with no obvious initiation sequence. Thus, the somatic and testis sites are apparently controlled by overlapping promoters with different properties.

We have looked for testis-specific transcription factors that would bind to the cAspAT promoter and would modify the transcription start sites pattern. We have not found any testis-specific footprint within the proximal 300 bp of the promoter. The same DNA sites are bound by proteins from the liver and testis. However, when these DNA-protein interactions were analyzed by gel retardation assays, significant differences appeared in both the relative mobility and the heat sensitivity. In the liver, the transcription factors binding to these DNA sites are members of the C/EBP family (8). It is likely that other members of this family are implicated in the testis. Whether these differences in DNA-protein interactions are responsible for the different pattern of transcription initiation has yet to be proven. It is still possible that this pattern results from differences in the transcription initiation machinery or from a different chromatin environment.

The mechanisms by which the multiple transcription start sites of housekeeping gene promoters and some other promoters are controlled remain poorly understood. In promoters comprising a TATA box, this element that binds the TFIIID factor determines the location of the transcription starts site (37, 38). In a second class of promoters with a single initiation site, the absence of a TATA sequence is compensated by the presence of “initiator elements,” which actually span the initiation site (5, 39, 40). It is thought that in the absence of an initiator or a TATA box, transcription initiates at several sites (41), despite the participation of the TFIIID factor to the transcription machinery (42). What controls the location of these sites remains elusive. In one example, the androgen receptor gene promoter, the Sp1 transcription factor was shown to be required for one of the transcription sites but not for the other (43). In the absence of data on the molecular mechanisms involved, the observations made here on the cAspAT gene transcription show that there is a tissue-specific as well as a developmental control of the pattern of initiation sites in a housekeeping gene promoter. In a previous study, we have shown that glucocorticoids differentially regulate the transcription initiation sites of the cAspAT promoter (3). Thus, the relative usage of the multiple transcription initiation sites is dependant on both hormonal and tissue-specific factors, a clear indication that those sites are not coordinately regulated. A similar observation was made on another promoter with multiple transcription start sites, the proenkephalin gene promoter (44).
What are the physiological consequences of the different pattern of transcription initiation in the cAspAT gene promoter? We have observed that the cAspAT activity is several fold lower in the testis as compared with the liver or kidney, while the total amount of mRNAs is not significantly altered. It is possible that the mRNAs in the testis are less efficiently translated. This could be due either to the polyadenylation pattern or to the initiation pattern. We cannot eliminate either possibility. However, in vitro translation studies performed on the human mRNAs have shown that the efficiency of translation is independent of the polyadenylation site (23). Furthermore, the upstream initiation site in the testis yields a mRNA with a high 5' GC content, a sequence that favors stable secondary structures, and thus may influence the efficiency of translation. If this is the case, the altered pattern of transcription initiation in the testis would account for the lower activity of the enzyme in this organ, a situation similar to that of the testis-specific far-neusyl pyrophosphate synthetase mRNAs (36).

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