Volume 14 Number 22 1986 Nucleic Acids Research

Adipsin, the adipocyte serine protease: gene structure and control of expression by tumor necrosis factor

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Received 15 August 1986; Revised and Accepted 20 October 1986

ABSTRACT

We have isolated, mapped and sequenced adipsin, the adipocyte differentiation-dependent serine protease gene. This gene, which is present in a single form in the mouse, spans 1.7 kilobases and contains five exons. While the basic exon structure characteristic of serine protease genes is conserved in adipsin, there is also a fusion of two exons that are separate in other serine proteases. The sequence data also suggests a mechanism of alternative splicing which appears to account for the generation of two adipsin mRNA species differing by only three nucleotides and encoding two different signal peptides. To investigate the control of adipsin expression we have examined the effects of tumor necrosis factor (TNF) on adipocytes. The level of adipsin RNA is dramatically decreased by hormone treatment, but the change occurs more slowly than for other fat cell mRNAs, such as glycerophosphate dehydrogenase. These results show that adipsin is a novel serine protease gene whose expression is regulated by a macrophage-derived factor which modulates expression of other adipocyte-specific RNAs.

INTRODUCTION

Our laboratory has been studying the molecular basis of cell differentiation using 3T3-F442A adipocytes as a model system. The conversion of 3T3-F442A preadipocytes to adipocytes in culture resembles *in vivo* fat cell development in many respects; in the process of differentiation, these cultured cells accumulate lipid droplets, exhibit hormone sensitivities appropriate for adipocytes, and increase synthesis of enzymes involved in fatty acid and triglyceride synthesis (1-7). The changes in protein biosynthesis accompanying adipocyte differentiation parallel alterations in levels of specific mRNAs (7,8). We have isolated several clones from cDNA libraries (8) corresponding to mRNAs that are induced during fat cell differentiation. One of these mRNAs encodes an *in vitro* translation product of 28,000 daltons and was shown by sequence analysis to be a novel member of the serine protease family (9). We previously called the encoded protein 28K and have since renamed it adipsin (10). It is primarily expressed in adipose tissue (9,11) although recent studies indicate that the sciatic nerve is also an important

site of synthesis (Cook, Min and Spiegelman, unpublished results). While the role of adipsin in adipose physiology is unknown, several features of adipsin primary sequence suggest that it is an active protease. It possesses the key catalytic residues necessary to make it an active enzyme; moreover, it has a potential cleavage site for activation of the zymogen (9).

Expression of the adipsin gene appears to be regulated in a manner that is unusual in several respects. First, transcription of the adipsin gene in the adipocyte appears to be much lower than expected from the abundance of this mRNA (12,13). A relatively slow turnover of this message, compared to other mRNAs in the fat cell appear to account, at least in part, for its great abundance (12). Furthermore, adipsin mRNA exists in two forms in the adipocyte, differing by deletion of 3 nucleotides which code for the terminal amino acid residue of the predicted signal peptide (9).

Because adipsin is a novel member of the serine protease family and has several interesting features to its regulation, we have isolated the mouse gene encoding this protein. Structure and sequence analysis has allowed us to compare this gene to other members of the serine protease family and propose a likely mechanism to account for the alternative splicing pattern. Furthermore, we have shown that expression of adipsin mRNA is regulated by the macrophage cytokine, TNF.

MATERIALS AND METHODS

Cell Culture and Isolation of RNA

3T3-F442A cells were grown as described (8). For experiments using TNF, cells were treated at confluence (Day 0) for 6 days or after differentiation (Day 5) for various times with 2500 U/ml recombinant TNF (Asahi Chemical Industry Co. Ltd., Japan) added to culture media. RNA was extracted from these cells using 5 M guanidine isothiocyanate and centrifugation through 5.7 M cesium chloride (14).

Construction and Screening of 3T3-F442A Adipocyte Genomic Library

The 3T3-F442A adipocyte genomic library was constructed in an EMBL4 replacement vector as described (15). Nick-translated adipsin cDNA plasmid (8) was used to screen the genomic library of $5x10^5$ recombinant plaques. Three positive clones were obtained in four rounds of plaque purification.

Southern and Northern Blot Analysis

Southern blots were performed as described in Maniatis et al. (16) with the exception that the radioactive probe for the inter-species blot was prepared by random priming of a cDNA fragment isolated from SeaPlaque agarose

(17). The mouse filter was washed at 65°C for 2.5 h with 0.1xSSC/0.5% SDS. The inter-species Southern filter was washed at 60°C for 1 hr with 2XSSC/0.1% SDS. RNA blots were run on formaldehyde gels, then transferred onto Gene Screen Plus (New England Nuclear), hybridized and washed using the protocol provided by the manufacturer. Probes were prepared by random priming of cDNA fragments for glycerophosphate dehydrogenase, aP2 and adipsin (8). The autoradiograms were quantitated by scanning densitometry.

DNA Sequencing of the Adipsin Gene

Two Bam Hl fragments of 1.8Kb and 1.4Kb in length, comprising the entire adipsin gene were cloned into M13mp18. The DNase deletion sequencing strategy of Hong (18) was employed, using [α-^{35}S]dATPαS. To clarify sequence situated 100 to 250 bp upstream of the polyadenylation addition signal, the chemical sequencing method of Maxam and Gilbert (19) was used.

RESULTS

Isolation and Mapping of the Adipsin Gene

A lambda clone (λ-ADN) spanning 13Kb and containing the adipsin gene was isolated and a restriction map was constructed (Figure 1). Hybridization of λ-ADN restriction fragments with adipsin cDNA plasmid (pAd-20) revealed a 6.2Kb EcoRl band, as well as 1.8Kb and 1.4Kb Bam Hl fragments. The 1.8Kb Bam Hl fragment resulted from the regeneration of a Bam Hl site at the left arm of the EMBL4 vector. Subsequent sequence analysis (Figure 3) showed that the 1.8Kb and 1.4Kb Bam Hl fragments contain the entire adipsin gene, including 0.9Kb of 5' and 0.7Kb of 3' flanking sequence. A detailed restriction map of this region is included in Fig. 1.

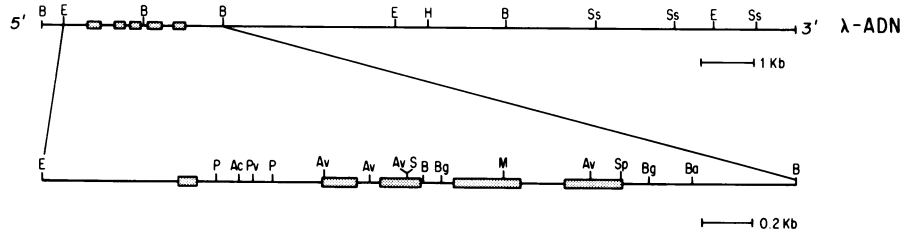

Figure 1. Adipsin restriction map and exon structure.
At top of figure is a restriction map of the λ-ADN clone, containing the adipsin gene. Shaded boxes indicate exons. Below, the region of the λ-ADN clone containing the adipsin gene sequence and flanking sequences is expanded to show a detailed restriction map. Ac=Acc I; Av=Ava I; Ba=Bal I; B=Bam HI; Bg=Bgl II; E=Eco RI; H=Hind III; M=Mst I; P=Pst I; Pv=Pvu II; S=Sma I; Sp=Sph I; Ss=Sst I; St=StuI.

Southern Blot Analysis of the Adipsin Gene in Mouse and Other Species

Genomic DNA from 3T3-F442A mouse preadipocytes and adipocytes were cut with EcoRI or Hind III and probed with nick translated adipsin cDNA plasmid. In both preadipocyte and adipocyte DNA, a 6.2Kb EcoRI fragment and a 10Kb Hind III fragment showed positive hybridization, consistent with the restriction map of λ-ADN (Figure 2A). This indicates that adipsin is present in a single

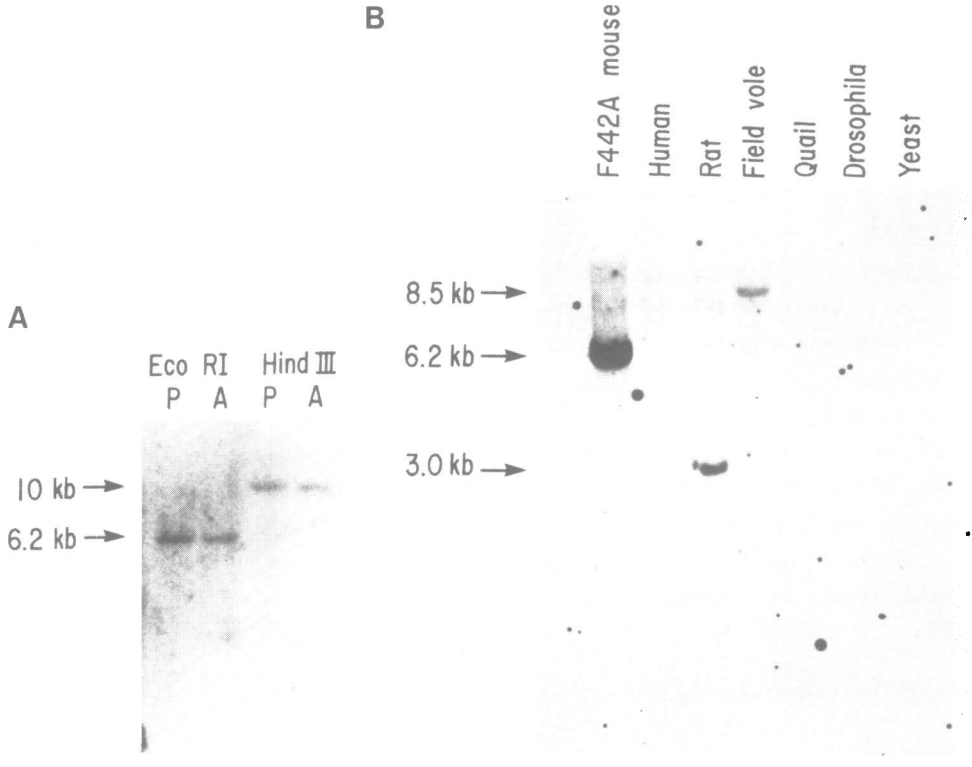

Figure 2. Genomic Southern bloting of adipsin gene.
Blotting and hybridization were done on nitrocellulose filters as described (16). (A) DNA from 3T3-F442A cells. P, preadipocyte DNA; A, adipocyte DNA. DNAs were digested with the enzymes indicated at the top of the panel. Probe was a cDNA plasmid (8) nick-translated to a specific activity of $3x10^8$ Cerenkov cpm/μg DNA. Washes were done at 65° C with 0.1x SSC and 0.5% SDS for 2.5h (1x SSC=0.15M sodium chloride, 0.015M sodium citrate, pH 7.0). Autoradiography was for 6 days with an intensifying screen. (B) DNA from various species, digested with EcoRI 20 μg of DNA was used, except for Drosophila (3 μg) and yeast (2.5 μg). Probe was a cDNA insert (8) labeled by random priming to a specific activity of $4.3x10^9$ Cerenkov cpm/μg DNA. Blot was washed for 1h with 2xSSC/0.1% SDS at 60°C. Autoradiography was for 4.5h with an intensifying screen.

form detectable in mouse, and that the gene does not undergo rearrangement during adipose differentiation. Southern blot analysis of genomic DNA from various species showed one strongly hybridizing band in rat and field vole DNAs (Figure 2B), and only very weak, but reproducible bands in human and quail DNA. No hybridization was evident with Drosophila or yeast DNA.

Sequence of the Adipsin Gene: Exon Organization

The sequence of the entire adipsin gene, including 0.5Kb of 5' and 0.7Kb of 3' flanking sequence, was obtained by the dideoxynucleotide chain termination method. The transcription start site was determined by using primer extension mapping data previously reported by our laboratory (9). This G residue is situated 19bp 5' of the ATG translation initiation codon, and 27bp downstream of a canonical TATA box, 5' CATAAAA 3'. The intron-exon boundaries were determined by comparing the adipsin genomic sequence to the cDNA sequence (9). All of the boundaries follow the GU...AG rule of Breathnach et al. (20) and agree well with consensus sequences for splice donor and acceptor sites (21). The adipsin gene is 1748 base pairs and consists of 5 exons of 74, 157, 148, 258 and 230bp, interrupted by introns of 475, 90, 141 and 175bp, respectively. A characteristic feature of serine protease genes, in which each active site residue is encoded by a separate exon, is conserved in the adipsin gene. Thus, His-66, Asp-115, and Ser-209 (equivalent to His-57, Asp-102 and Ser-195 in chymotrypsin; see 9) are encoded separately by exons 2, 3 and 5. Also, as is typical in serine protease genes, exon 1 in adipsin includes the 5' untranslated region and the putative signal peptide.

Proposed Alternative Splicing Mechanism for Generation of Two Signal Peptides

We previously reported the existence of two different adipsin mRNAs of equal abundance in 3T3-F442A cells, differing only by an insertion of a trinucleotide, C-A-G, within the 19th codon. Because the sequences of these two RNAs were otherwise identical, we proposed that the two species arose by alternative splicing of a primary transcript containing two adjacent splice acceptor sites (9). Genomic sequence provides evidence that two potential splice acceptor sites do exist at the Intron 1-Exon 2 boundary (Figure 4). Both of these sites follow the GU...AG rule and agree closely with the consensus splice acceptor sequence (21). Since the splice sites occur at the 3' end of the sequence encoding the putative signal peptide, the utilization of the alternative splice acceptors would result in two different signal peptides differing only by the addition of alanine at the end of the longer signal sequence.

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GGAAATGAAACCTCATGGCAACACTCTGCTTGCTGAGGTGTGAGACCCCACAGACACAGG  -442
AATGAGGACAAAGGAGTTAAGTGCCCTTCCCCCACTGTCAAGCAACCTTCTCTCCAAATT  -382
GAAGGCAGAAAGAGGAGGGAGAGATGATGGGGTGGAGGGTGTTACTAACCGACCTCCTGC  -322
TGCTCTGGTTTTAACAGACACCATGACAGACACATGGCTGGAAGGATACTCACTGGGCCA  -262
GGACAACAGGTGGTCCATCCTCTGCTCTCGTTGTGCCTTGTGTATGCCTACCTTGAGAGT  -202
GGGCACTGGTCAGGGTGCCATGCAGGAGGGAGAGGAGGGTTTGCCCTGCCCCCTGCACCT  -142
CACAGGTTCACATCATCTGTCCCAGAAGGGCTGACACACCGAAAGTGCAGACTCCCCTTC   -82
CCTAGTTGGTTTTCTGCCCACCAGGCAAGGGGCAGGAGGTAAGAGGCAGGAGTCCATAAAA  -21

                                        EXON 1
                          +1            MetHisSerSerValTyrPhe
CAGCCCTGAGAGCCTGCTGG  GTCAGTGCCTGCTGTCAGA ATGCACAGCTCCGTGTACTTC  40

ValAlaLeuVal IleLeuGlyAlaAlaValCysA INTRON 1
GTGGCTCTGGTGATCCTGGGAGCGGCTGTATGTG GTGAGTGGACCAGGGAGGGACCAGTT  100

TGACACAGCAGCCCCCTTGGTGGGCCAGCACAGCAGGAGTCCCCTGCAGTGAAACACTCT  160

CCAAATTACTAATCTGTAATCTGAGCCTTCTGGTTTCAGAGCTTTTATCAACAGCAAGAA  220

TCTGGATTTGAAATCTGTATACTTTACAGAATTAAAGAACTAAAATTCACCTTAGGGCTA  280

GCGAGAAGGGTCAGCTGGTTAAAGGGCCTGAACACCCATTTGATGAAGAAAGTGATCGCT  340

ACAAGTTGTCTTCAGCCTACTGCAGGTTCACTGTGGTCTTGAGGGGTGTAAACCACCCAC  400

AGGCCCCTGAATTTGCAGCCCCTCGGAAATGCACTTTACTGAGATCGCTTTTGGGTCTGG  460

GGCAACTGGGTTGGAGGTGTCTGTGGTCAGAGTGGGTTCTCTTTAGGTGCAACCCACCCC  520

                                  EXON 2
                                  laAlaGlnProArgGlyArgIleLeuGly
AACCCTTACAATACACCTACCCTCCTCAG CAGCACAGCCCCGAGGCCGGATTCTGGGT    578

GlyGlnGluAlaAlaAlaHisAlaArgProTyrMetAlaSerValGlnValAsnGlyThr
GGCCAGGAGGCCGCAGCCCATGCTCGGCCCTACATGGCTTCCGTGCAAGTGAACGGCACA  638

HisValCysGlyGlyThrLeuLeuAspGluGlnTrpValLeuSerAlaAlaHisCysMet
CACGTGTGCGGTGGCACCCTGCTGGACGAGCAGTGGGTGCTCAGTGCTGCACACTGCATG  698

AspGlyVa INTRON 2
GATGGAGT GTGAGTGGCAGGGGTGGGATGGGGGTGGGAGGAGCGGGGCCTCGGGAGGCTG  758

                                        EXON 3
                                        lThrAspAspAspSerValGln
GGACGTGGATCTGAGATGCGCCCTTGTCCTGTCCCCAG GACGGATGACGACTCTGTGCAG  818

ValLeuLeuGlyAlaHisSerLeuSerAlaProGluProTyrLysArgTrpTyrAspVal
GTGCTCCTGGGTGCCCACTCCCTGTCCGCCCCTGAACCCTACAAGCGATGGTATGATGTG  878

GlnSerValValProHisProGlySerArgProAspSerLeuGluAspAspLeuIleLeu
CAGAGTGTAGTGCCTCACCCGGGCAGCCGACCTGACAGCCTTGAGGACGACCTCATTCTT  938

PheLys INTRON 3
TTTAAG GTGAGAGAGCTGGATCCTTTGTACTCAAGAGTCTAGCCTCGACCCTACCTTCTG  998

CACCAGAATCCCTGTCCGCCACCCCAGCTTCAGATCTCTTTTCTTGACTCTGCCCCTACT  1058

                                  EXON 4
                                  LeuSerGlnAsnAlaSerLeuGlyProHisVal
CAGACATTCTGCCCCTCCCACCCTTAG CTATCCCAGAATGCCTCGTTGGGTCCCCACGTG 1118

ArgProLeuProLeuGlnTyrGluAspLysGluValGluProGlyThrLeuCysAspVal
AGACCCCTACCCTTGCAATACGAGGACAAAGAAGTGGAACCCGGCACGCTCTGCGACGTG  1178

AlaGlyTrpGlyValValThrHisAlaGlyArgArgProAspValLeuHisGlnLeuArg
GCTGGTTGGGGTGTGGTCACCCATGCAGGACGCAGGCCTGATGTCCTGCATCAACTCAGA  1238

ValSerIleMetAsnArgThrThrCysAsnLeuArgThrTyrHisAspGlyValValThr
GTGTCAATCATGAACCGGACAACCTGCAATCTGCGCACGTACCATGACGGGGTAGTCACC  1298

IleAsnMetMetCysAlaGluSerAsnArgArgAspThrCysArg INTRON 4
ATTAACATGATGTGTGCAGAGAGCAACCGCAGGGACACTTGCAGG GTGAGGGACACAGTC 1358

TGGGTGATGTGGTGTGGGTGGGTGGAGTTGAGGGGCTGTGGAGGCTAGCTAGGGGGCCTG  1418

GCTTGTGGGAAGGGTGGGGTTCAGGAATCTCAGGAGCTTCTGGGAGTCAGTTTCTTGCTG  1478

                                        EXON 5
                                        GlyAspSerGlySerPro
GCTATTGGGTGGGCATGTCACTAGTGGCCCTGCCCTACAG GGAGACTCCGGCAGCCCT   1536

LeuValCysGlyAspAlaValGluGlyValValThrTrpGlySerArgValCysGlyAsn
CTAGTGTGCGGGGATGCAGTCGAAGGTGTGGTTACGTGGGGCTCTCGCGTCTGTGGCAAT  1596

GlyLysLysProGlyValTyrThrArgValSerSerTyrArgMetTrpIleGluAsnIle
GGCAAAAAGCCGGGCGTCTATACCCGAGTGTCATCCTACCGGATGTGGATCGAAAACATC  1656

ThrAsnGlyAsnMetThrSer .
ACAAATGGTAACATGACATCCTGA GGGGACACCAGAGACACGTGGCTCAGGGAAACAAGA 1716

GACACGTGGCGCACAATAAATGCATGCATCTG▼AGCCCTGTGCATCTTTCTTTTTTTTTTT 1776
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TTTAAGATTTATTTATTGTTATATGTATCTACACTATAGCTGTCTTCAGACACACCAGAA  1836
GAGGGAGTCAGATCTTATTACAGATGGTTGTGAGCCACCATGTGGTTGCTGGGATTTGAA  1896
CTCAGGGCTTCGGAAGAGCAGTCAGTGCTCTTAACCACTGAGCCATCTCACCAGCCCGCC  1956
CTGTGCATCTTTATTGAGTGCCTGTTGCATACACACCTGAAGGCGGGTGTTTTCACGCTT  2016
GGCCACACGGGGGCGCGCGTGCTGTTTTAGAATTGTGAGGCGGGGCTCATTCCTATCCTA  2076
CTGAGGTCCTGCTGTACCCAGGACTGTTGTCTGGGCTCAGACAGCTCTTACTTTGAGCCT  2136
TACCACATAAATACTAAAAGGGAAAACTGAGGCACAGAGCGGCCATGAACAAGACTGGAT  2196
TCCAACCCAGAGCCCTTCCTGGTTTACTGAAGAGGGAGTTGGTAATGTCTTTGTCACAGG  2256
CTGGAACCTGTGGGTGACAATGGGCAGAACCTGGACCCAAACCCTGTGTCTTTCTACCAG  2316
GAGAACAGGACACTGAGGCAAAGGGAGTCTTCTGGCTTCCTGTAGGTATTGGAGACTAAG  2376
GATCTTAGAGGTGAAACCACTGAGGACCCTGCTGTGAAAGAGGATAGAACTGGATCC     2433
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Figure 3. Nucleotide sequence of adipsin gene.
Start of transcription is indicated by +1. A canonical TATA homology at -27 relative to the start site and the polyadenylation addition signal at +1731 are boxed. Arrow indicates the site of poly(A) tail insertion. Regions of internal homology and dyad symmetry (see Results) in the 5' flanking region are underlined.

Promoter Sequences of Adipsin Gene

A canonical TATA box, 5' CATAAAA 3', is located 27bp upstream of the transcription start site. In addition, a computer-assisted search of the upstream region of the adipsin gene revealed several noteworthy structural features. First, there is an internal homology element at positions -308 to -286 relative to the transcription start site which consists of a 12bp tandemly repeated sequence with one base mismatch: 5' ACAGACACCATG/ACAGACACATG 3' Secondly, at nucleotides -178 to -163, there is a directly repeated 7-mer AGGAGGG, with 2 nucleotides, AG, between the repeats (AGGAGGGAGAGGAGGG). The repeated 7-mer, is also seen at position -369. Seven nucleotides of the longer sequence, GAGAGGA, overlaps the 15 base FSE2 putative regulatory element that we previously described as being present in two other adipose differentiation-specific genes, aP2 and glycerophosphate dehydrogenase (10).

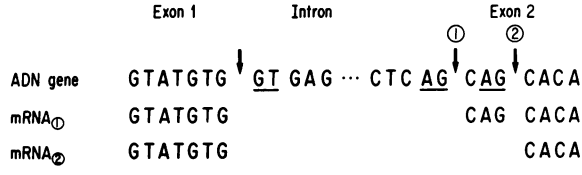

Figure 4. Proposed mechanism for alternative splicing of the adipsin transcript.
The sequence of the splice junctions between exon 1 and intron 1, and intron 1 and exon 2 are shown. Consensus sequences for splice donor and acceptor sites are underlined. Arrows marked 1 and 2 indicate the proposed splice acceptor sites which would generate the corresponding mRNAs 1 and 2.

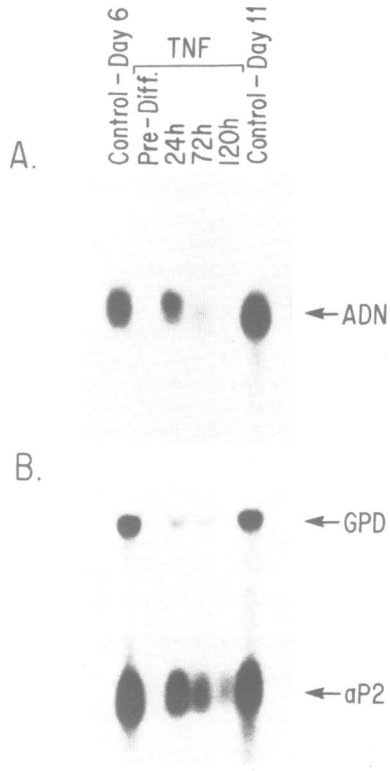

Figure 5. Northern blots showing the effect of tumor necrosis factor on Adipocyte RNAs.
3T3-F442A cells were grown to confluence (Day 0), and allowed to differentiate until Day 5, when 90% of the cells had undergone adipose conversion. Beginning on Day 5, the adipocyte cultures were treated with 2500 U/ml recombinant TNF for 24, 72 or 120 hours, and harvested for RNA extraction. Untreated adipocytes were harvested on Day 6 and Day 11. "Pre-diff." indicates treatment with 2500 U/ml TNF from Day 0 to Day 6 which blocked adipose conversion. (A) Blot probed with adipsin cDNA. 10 µg total RNA was loaded in each lane. Autoradiography was for 5h with intensifying screen. (B) Blot probed with a mixture of glycerophosphate dehydrogenase and aP2 cDNAs at a ratio of 15:1 cpm/ml (GPD:aP2). 20 µg total RNA was loaded in each lane. Autoradiography was for 1h with intensifying screen.

Finally, there is a region of dyad symmetry at nucleotides -90 to -73 and -65 to -47 which could potentially form a hairpin loop 19 base pairs long.

Effect of Tumor Necrosis Factor on Adipsin Gene Expression

We are interested in those agents which may regulate the expression of the adipsin gene. Because it has been recently reported that cachectin (now known to be identical to TNF) (reviewed in 22) rapidly affects the expression

of several adipocyte-specific mRNAs (23), we examined the effect of TNF on adipsin mRNA levels. 3T3-F442A cells were treated with 2500 U/ml TNF before or after undergoing adipose differentiation. When TNF was added to confluent 3T3-F442A preadipocyte cultures for 6 days, morphological conversion to adipocytes was totally inhibited (not shown) and the accumulation of adipsin mRNA was completely blocked (Fig. 5). The addition of TNF to mature adipocytes for 1 to 5 days resulted in a slow and gradual loss of lipid droplets from the adipocytes, so that after 5 days of TNF treatment, the number of cells possessing lipid droplets had been reduced by one-half. As previously noted (23) and confirmed here, GPD mRNA is rapidly lost from TNF-treated cells, decreasing by greater than 90% in cells treated for 24 hrs with TNF. Adipsin mRNA level was also decreased in TNF-treated adipocytes, but this decrease occurred considerably more slowly, with 44% of adipsin RNA remaining after 24 hrs of hormone treatment. aP2 mRNA was reduced to 55% of its control level with the same treatment. The Day 11 control lane shows that the level of all three RNAs stays high throughout the experiment in untreated cells. A control blot (not shown) confirmed that TNF does not cause a decrease, but actually causes a slight increase, in actin RNA, verifying that hormone treatment of adipocytes does not result in non-specific degradation of all RNAs in the cell. These results show that TNF causes a very large reduction in all three adipose RNAs studied here. However, adipsin and aP2 mRNA appear to be reduced more slowly than GPD and the other adipose-specific mRNA studied previously.

DISCUSSION

Adipsin is a member of the serine protease gene family whose expression is largely limited to adipose tissue in the mouse. It is induced at least 100-fold during mouse 3T3-F442A adipocyte differentiation and encodes a novel serine protease homologue which has the key features necessary to make it an active enzyme (9). Because this represents the first identification of an adipocyte-specific serine protease, and one of only a few members of this family analyzed which is distinct from the pancreatic and blood clotting enzymes, it was of great interest to compare the structure and sequence of the adipsin gene to that of other serine protease genes. In these comparisons, striking similarities as well as intriguing differences are evident. One characteristic feature of serine protease genes is that components of structural and functional domains are encoded by separate exons. Thus, the active site residues that form the charge relay system, His-57, Asp-102, and

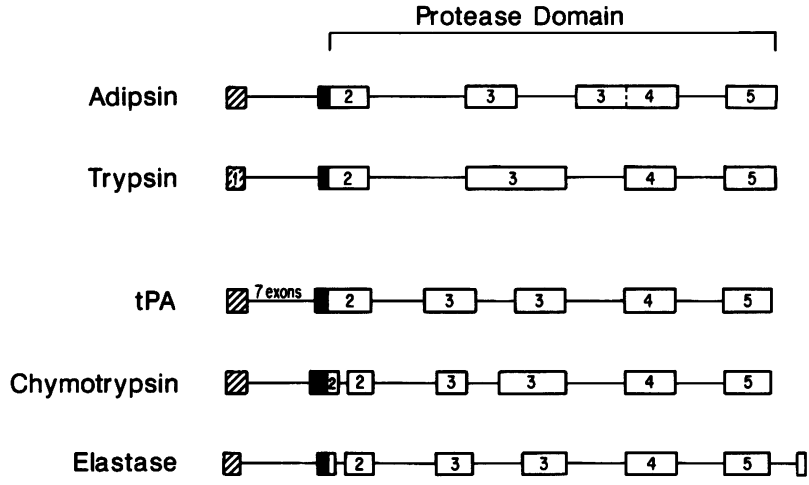

Figure 6. Comparison of adipsin exon structure to other serine protease genes.
Boxes indicate exons, and are drawn to relative scale. The lines between the boxes indicate introns; the lengths of the lines do not correspond to relative intron sizes. Comparisons of exon structures are made relative to trypsin so that the five exons of trypsin are numbered 1 to 5, and the exons of the other genes are numbered according to their homology to the exons in trypsin. The slashed boxes represent the signal sequence of each gene, and the filled boxes indicate portions of the serine protease genes that bear no homology to each other (27). The dotted line within the fourth exon of adipsin indicates portions which bear homology to the 3rd and 4th exons of trypsin.

Ser-195 in chymotrypsin, are separately encoded in serine protease genes; the adipsin gene is no exception, having these residues encoded by exons 2, 4 and 5, respectively. Furthermore, in adipsin as in other serine proteases, the first exon encodes 5' untranslated leader and the signal peptide. Therefore, the basic organization of exons typical of the serine protease gene family is conserved in adipsin. There is one aspect of exon structure in which the adipsin gene clearly diverges from other members of the serine protease family, as illustrated in Figure 6. In this figure, the exons in each gene are numbered in correspondence to their homology to the exons in trypsin. It is apparent that adipsin is unique among the serine protease genes in having lost the intron between exons 3 and 4, so that the latter half of exon 3 and exon 4 have fused in relation to trypsin. However, intron 3 is still positioned to interrupt the coding sequence at amino acids corresponding to those which map to the surface of the protein structure in other serine proteases, as has been noted (24). Finally, it is interesting to note that adipsin is the most compact gene among serine proteases depicted in Figure 5;

adipsin spans only 1.7kb, compared to 3.3kb in rat trypsin I (25), the smallest of the pancreatic serine protease genes studied, and 12kb in rat elastase I (26), the largest of these genes. Thus, we conclude from our analysis that the adipsin gene retains the characteristic feature of separating the catalytic residues into individual exons; however, adipsin is unique among the serine protease genes in having excised one common intron boundary. Although the functional and evolutionary significance of these structural similarities and dissimilarities are not known, it may prove enlightening to extend these comparisons as additional serine protease genes are discovered. The structure and sequence of mouse adipsin has also recently been determined by others (27).

In addition to comparing the structure of the adipsin gene to that of other serine protease genes, we were interested in finding possible common regulatory sequences among those genes. We focused on the mouse serine protease genes (28-30) to avoid cross-species comparisons since other available sequences have come mostly from rat (25,26,31,32) and the adipsin gene originated from mouse. Upon comparing 500bp of mouse glandular kallikrein sequence (28) upstream of the transcription start site to the entire adipsin gene sequence, we found two stretches of extraordinary homology. One of these is a 15bp purine-rich sequence, 5' CAGGACAGAGGAGGG 3'. The corresponding sequence from the adipsin gene, at position -377 relative to the start site, 5' CAGAAAGAGGAGGG 3', matches at 13 out of 15 nucleotides with a one nucleotide gap. Homologous sequences are also found in the upstream regions of three other mouse glandular kallikrein genes, α-NGF, γ-NGF, and renal kallikrein; however, their homologies to the adipsin -377 sequence are at best 11 out of 15 nucleotides. Interestingly, a portion of this sequence, 5' AGGAGGG 3' is repeated three times in the 5' flanking region of the adipsin gene. A second sequence, forming perfect 11 nucleotide match, 5' GAGCCTTCTTG 3' is seen 157bp upstream of the kallikrein TATA box and in the first intron of the adipsin gene (+183). α-NGF, γ-NGF, and renal kallikrein contain homologous sequences of 8 or fewer nucleotide matches out of 11. The 5' flanking regions of rat elastase, chymotrypsin and trypsin genes (25,26,31) do not contain homologies to either of these sequences. It is not yet known whether either of the above sequences constitutes a regulatory element in the adipsin and kallikrein genes; nevertheless, these sequence homologies are interesting in view of the fact that neither sequence resides in coding regions of these genes, and therefore do not represent sequence conservation in the protease domain of this gene family.

The sequence data presented here also provides an explanation for the generation of the two forms of adipsin mRNA, which differ in length by 3 nucleotides. We previously suggested that the deletion of a CAG codon, which encodes the terminal amino acid of the predicted signal peptide, occurred via alternative splicing. The results here strengthen this hypothesis and suggest a mechanism. Two potential splice acceptor sites are located three nucleotides apart at the end of the first intron. Depending on which acceptor is employed, an additional alanine residue may or may not be included at the terminus of the signal sequence. Recent work in our laboratory (Cook, Min and Spiegelman, unpublished results) has shown that the adipsin protein is present in two distinct forms, both of which are secreted. Whether these forms bear any relationship to the alternative splicing of the adipsin mRNAs must await protein sequence analysis.

The factors which modulate adipsin gene expression are unknown. Of particular interest to us as a potential modulator was tumor necrosis factor, a hormone produced by macrophages in response to endotoxin. Torti et al. (23) showed that cachectin, which has subsequently been shown to be indentical to tumor necrosis factor (33,34,22), caused a rapid disappearance of adipocyte-specific RNAs from mature TA1 fat cells in culture, so that by 12-24h of treatment with cachectin, their adipocyte-marker RNAs were diminished by 90% of their control level. In light of these results, we wished to investigate whether the level of adipsin RNA in 3T3-F442A adipocytes would be affected to a similar extent and with a comparable time course as the adipocyte-specific RNAs examined by Torti et al. Our results indicate that tumor necrosis factor does indeed reduce the level of adipsin RNA in 3T3-F442A adipocytes; however, the effect of TNF on adipsin RNA occurs rather slowly, with 44% of adipsin RNA remaining after 24 hrs of treatment. We then examined the time course of TNF effect on two other adipocyte differentiation-dependent RNAs, aP2 and glycerophosphate dehydrogenase (GPD). While aP2 RNA also decreased slowly, GPD RNA disappeared in the same rapid time scale as that seen by Torti et al. (23), making it unlikely that the more gradual depletion of adipsin and aP2 RNA were due to differences in the cell lines or hormone preparations used in the two studies. One possible explanation of these results may lie in the rates of turnover of the different RNAs. Previous work from our laboratory using the transcription inhibitor DRB showed that in 3T3-F442A adipocytes, GPD is a relatively short-lived mRNA, with a half-life of 2h, whereas aP2 and adipsin have longer turnover times of 12h and >30h, respectively (12). While these turnover rates must be considered as only preliminary because of

possible secondary effects of the transcription inhibitor itself (35), the general correspondence between turnover of the RNA in the presence of DRB and loss due to TNF is consistent with the notion that TNF may function primarily by shutting off transcription and that the rates at which specific mRNAs are lost are linked to their normal turnover rates. While their transcription studies were done in cells treated with TNF before differentiation, Torti et al. have previously suggested a transcriptional mode of action for TNF (23).

The general sensitivity of fat cell-specific mRNAs to suppression by TNF suggests that this agent acts on these genes at a site or sites which is both of fundamental regulatory importance and common to most if not all fat cell-specific genes. Our comparison of promoter sequences of three genes specifically activated in fat cells indicated that there are indeed 2 common sequence elements, which also show homology to known viral enhancer core elements (10). In light of the existence of these "fat-specific elements" (10), we speculate that TNF may influence interactions with one or both of these putative regulatory elements or other sequence elements common to fat-specific genes, either directly or with nuclear proteins that bind to these elements. Studies are underway in our laboratory to address these hypotheses.

ACKNOWLEDGEMENT

This work was supported by grants from the N.I.H. (AM31405 and AM34605).

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