We have isolated and sequenced a genomic clone coding for the first three exons and the 5'-flanking region of the human fatty-acid synthase gene. The translation initiation site, ATG, is located in exon I. Primer extension and S1 nuclease analyses showed the presence of three transcription initiation (Ti) sites: Ti I, Ti II, and Ti III. The Ti I site is mapped to the beginning of the untranslated exon I and preceded by a promoter with recognizable TATA and CAAT boxes. The Ti II and Ti III sites are located in intron I, at 60 and 49 nucleotides upstream of the translation initiation site ATG in exon I, respectively. These two Ti sites are preceded by four putative Sp1 boxes, but lack TATA and CAAT boxes. Analysis of luciferase reporter gene expression in transient transfection assays confirmed the existence of two promoters. A 200-base pair 5'-flanking region, which has strong promoter activity comparable with that of the CMV promoter, is considered human fatty-acid synthase promoter I. In a wild-type human fatty-acid synthase-luciferase construct, in which promoter I and intron I are present in their natural configuration, the reporter gene activity is only 1% of that of promoter I. Deletion analysis showed the existence of promoter II, which is located in intron I immediately upstream of the Ti II site. The strength of promoter II is approximately 1/15th of that of promoter I in transient transfection assays. Further analysis of reporter gene constructs showed that promoter II inhibited the reporter gene activity of the wild-type construct that contained promoter I and intron I and that the spatial separation of the two promoters is important for this inhibition. A model is proposed based on the possibility that the assembly of transcription complexes on promoter II creates a "roadblock" and reduces the overall expression of the fatty-acid synthase gene by interfering with the progression of transcription from promoter I.

Although people are becoming conscious of limiting their intake of dietary fat, our fundamental knowledge of the complex set of interactions between dietary intake, hormonal stimulation, and gene regulation of the de novo synthesis of fatty acids in humans is very limited. Long-chain fatty acids are the major constituents of fats and lipids and play an important role in obesity and hyperlipidemia. In humans, as in other animals, fatty acids are either derived from food or synthesized de novo from acetyl-CoA by means of a complex set of reactions that are catalyzed by two multifunctional enzymes, acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FAS; Refs. 1 and 2). ACC, a biotin enzyme, catalyzes the formation of malonyl-CoA from acetyl-CoA. FAS catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH (1, 2).

Little is known about the regulation of FAS in humans. There is, however, ample evidence that the synthesis of FAS in animals is a well regulated process that is affected by diet and hormones at all stages of life, even during neonatal development and differentiation. A fat-free diet induces the synthesis of FAS and, consequently, an increase in the synthesis of long-chain fatty acids (2, 3). Starvation or diabetes mellitus represses the expression of the FAS gene. In fact, a dietary cycle of starvation and refeeding of a fat-free diet has been used routinely to enrich FAS in animal tissues, especially for the purposes of isolating the enzyme from liver tissue (1).

FAS levels are controlled by the rate of transcription and the stability of its mRNA (3–7). Hormones, such as triiodothyronine (T3) and insulin, affect the expression of the genes of many lipogenic enzymes, including FAS (8, 9). In primary cultures of chicken hepatocytes (8) and of mouse 3T3-L1 adipocytes (9), adding T3 to the culture medium caused a 2–4-fold increase both in the FAS mRNA level and in enzyme activity, whereas adding insulin alone had little or no effect. When T3 and insulin were added together, however, the response to T3 was amplified by 2-fold (8). Interestingly, the excess T3 produced in hyperthyroidism reduced the levels of FAS transcription (10).

The sequences of the regulatory regions in the rat and goose FASs contain several potential transcriptional regulatory elements, including hormone response elements (11–13). Recently, two different insulin response elements were identified in the regulatory region of rat FAS; one lies within a DNase hypersensitive region (14) and the other overlaps a CAAT box region (15). Regulation of the rat FAS reporter gene by diet and hormones has also been demonstrated by using transgenic mice (16). Interestingly, the CAAT box upstream of the TATA box with a CATGTT motif in the rat FAS sequence is an E-box sequence and serves as an insulin response element (15), a binding site of upstream stimulatory factor (17), and as a sequence required for the regulation of FAS by steroids (18, 19).

It is generally presumed that FAS is highly repressed in humans (20–22). It has been shown, however, that progestin increases and antiprogesterin (RU 486) decreases the transcription of FAS mRNA in the mammary tumor cell line MCF-7. In addition, RU 486 stabilizes FAS mRNA (23). Semenovich et al.
showed that the 2–5-fold increase in the FAS mRNA level in HepG2 cells that was induced by glucose occurred because of the increased stability of FAS mRNA. We have shown recently that although FAS mRNA is present in several human tissues at low levels, it is most abundant in brain, liver, and lung tissues (25). The tissue-specific distribution of human FAS mRNA suggests that the human FAS gene is regulatable. Although the hormonal regulation of the rat and goose FAS genes is being studied, information on the human FAS gene and its regulation will be more relevant to our long-term goal of studying the involvement of long-chain fatty acids in human diseases.

To study the regulation of the human FAS gene, we cloned and sequenced a 6-kb genomic DNA fragment that contained the 5′-flanking region of the human FAS gene and mapped the transcription initiation sites. In this communication, we report the molecular cloning of the gene, the identification of its two transcription initiation sites, and the analysis of the promoter functions. In addition, we report that transcription from the upstream promoter is blocked by the intron promoter, resulting in reduced overall expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—A λ-DASH human liver genomic DNA library was purchased from Clontech, Inc. (Palo Alto, CA). The radioactive nucleotides [γ-32P]dATP and [α-32P]dATP were purchased from Amersham Corp., and [α-32P]dCTP was purchased from DuPont NEN. The DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA) and U. S. Biochemical Corp. The Bst DNA polymerase together with Taq DNA polymerase was purchased from Perkin-Elmer. The HepG2 cell line was provided by Dr. G. J. Darlington (Department of Pathology, Baylor College of Medicine). The T47D cell line was obtained from the American Type Culture Collection (Rockville, MD). The cell culture media, the nick translation kit, and the LipofectAMINE transfection kit were purchased from Life Technologies, Inc. The calcium phosphate transfection kit was purchased from Invitrogen, Inc. (San Diego, CA). The Monolight 2010 Luminometer and the luciferase assay kits were purchased from Analytical Luminescence Laboratory (San Diego, CA).

**Isolation and Characterization of Genomic Clones**—The human liver genomic DNA library was screened at a density of 3,000 plaques/90-mm plate by using a λ-[32P]dCTP-labeled 485-bp human FAS cDNA fragment. The gel slab was dried and autoradiographed as described previously (26). Appropriate plaques, approximately 5 × 105 plaques, were picked. The phage DNA from the purified clones was isolated by using a KS(+) plasmid and sequenced with the vector primers and with the primers generated according to the method of Berk and Sharp (29). The hybridization products were digested with either restriction enzymes or Bal-31 exonuclease (30). The Bal-31-processed DNA fragments were blunted by using the Klenow fragment of DNA polymerase I (27) and were cloned upstream of the luciferase gene in vector pXP1. The human FAS promoter I-luciferase construct, LC9, contained a 926-bp FAS gene fragment containing the 3′-end of intron I and was tested upstream of the luciferase gene of vector pXP1 together with a 17-bp PstI-HindII adapter containing the natural 3′ splice site of human FAS (LC1). A 3.3-kb KpnI-KpnI human FAS gene fragment (nucleotides −1906 to +1394) immediately upstream of KpnI (nucleotides +1394) was isolated from AE6 and cloned at the unique KpnI site in LC1, producing the construct LC20. LC20 was further modified to construct LC20T, in which that the constant domain of LC9 by deleting a 1-kb SstI fragment from the 5′-end to generate LC3. LC3 contained promoter I, exon I, intron I, and the natural intron I splice sites. All the reporter gene constructs we created used the translation initiation codon ATG of the luciferase gene.

**Cell Culture Media and Conditions**—The human hepatoblastoma cell line HepG2 was maintained in minimal essential medium and Waymouth's MAB medium (3:1), the human breast cancer T47D cell line was grown in RPMI 1640 medium, and the human fibroblast cell line HT10-80 and HeLa cells were maintained in Dulbecco's modified Eagle's medium. The media were supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 100 milliliters/ml of penicillin and streptomycin. The cells were incubated in a humidified atmosphere of 5% CO2 at 37 °C. The medium was replaced with fresh medium twice a week and split 1:10 every week.

**Transient Transfection of HepG2 and T47D Cells**—The supercoiled plasmid DNA used for transfection was prepared according to the modified alkaline lysis method and was purified by two rounds of CsCl density gradient centrifugation (27). The cells were plated at a density of approximately 5 × 104 cells/cm2 and were transfected with 0.2 μg/cm2 plasmid DNA by using either a calcium phosphate transfection kit or a LipofectAMINE transfection kit. To normalize the efficiencies of transfection, CMV-β-gal DNA was cotransfected with the test plasmids at 500 the amount of test DNA. Sixteen hours after transfection, the cells were washed with culture medium. When the calcium phosphate transfection procedure was used, the cells were treated with culture medium containing 10% glycerol for 5 min before washing. The cells were fed fresh media and 48 h later were harvested and lysed. The luciferase activity was measured in relative light units by using an Enhanced Luciferase assay kit and a Monolight 2010 Luminometer. The β-galactosidase activity was measured as described previously (32, 33). To normalize the transfection efficiencies, the luciferase activity was expressed as a ratio of relative light units to the β-galactosidase activity obtained from the same extract. The luciferase activities obtained from the two different transfection methods were normalized by using the same constructs in both procedures.

**Misadventures Procedures—RNA isolation**—The 3′ untranslated region of the human FAS gene was cloned and sequenced using a plasmid vector and with the primers generated according to the method of Berk and Sharp (29). The hybridization products were digested with either restriction enzymes or Bal-31 exonuclease (30). The Bal-31-processed DNA fragments were blunted by using the Klenow fragment of DNA polymerase I (27) and were cloned upstream of the luciferase gene of vector pXP1 together with a 17-bp PstI-HindII adapter containing the natural 3′ splice site of human FAS (LC1). A 3.3-kb KpnI-KpnI human FAS gene fragment (nucleotides −1906 to +1394) immediately upstream of KpnI (nucleotides +1394) was isolated from AE6 and cloned at the unique KpnI site in LC1, producing the construct LC20. LC20 was further modified to construct LC20T, in which that the constant domain of LC9 by deleting a 1-kb SstI fragment from the 5′-end to generate LC3. LC3 contained promoter I, exon I, intron I, and the natural intron I splice sites. All the reporter gene constructs we created used the translation initiation codon ATG of the luciferase gene.

**RESULTS**

**Sequence Analysis of a Human FAS Genomic Clone Containing the 5′-Flanking Region of the Human FAS Gene**—A 6-kb genomic DNA fragment containing the 5′-flanking region of the human FAS gene was isolated and cloned into pbLueScript KS(+) vector, as described under "Experimental Procedures." This 6-kb EcoRI DNA fragment, called AE6 (Fig. 1), was digested with PstI and Smal restriction enzymes, and the fragments were subcloned into pbLueScript KS(+) plasmid and sequenced with vector primers and with the primers generated according to the method of Berk and Sharp (29). The hybridization products were digested with either restriction enzymes or Bal-31 exonuclease (30). The Bal-31-processed DNA fragments were blunted by using the Klenow fragment of DNA polymerase I (27) and were cloned upstream of the luciferase gene of vector pXP1 together with a 17-bp PstI-HindII adapter containing the natural 3′ splice site of human FAS (LC1). A 3.3-kb KpnI-KpnI human FAS gene fragment (nucleotides −1906 to +1394) immediately upstream of KpnI (nucleotides +1394) was isolated from AE6 and cloned at the unique KpnI site in LC1, producing the construct LC20. LC20 was further modified to construct LC20T, in which that the constant domain of LC9 by deleting a 1-kb SstI fragment from the 5′-end to generate LC3. LC3 contained promoter I, exon I, intron I, and the natural intron I splice sites. All the reporter gene constructs we created used the translation initiation codon ATG of the luciferase gene.
from the sequences obtained (Fig. 1). We overlapped the sequences of the subclones by sequencing DNA fragment AE6 with appropriate internal primers. The overall sequence is very rich in GC and had to be sequenced at 65°C by using Bst DNA polymerase and 7-deaza-DGTP in order to eliminate secondary structural artifacts. Comparing the sequence of AE6 with the sequences of the human FAS cDNA (25) and of the rat FAS genomic DNA (12, 13) revealed the presence of a putative 5'-untranslated exon and two translated exons (Fig. 1). The translation initiation codon ATG is located in exon II. Only a portion of the sequence of AE6 is shown in Fig. 2. The transcription initiation sites (Fig. 2) were later determined by primer extension and S1 mapping studies. The sequences of the human FAS exons II and III are, respectively, 98 and 90% identical to the sequence of the corresponding rat FAS exons. Both the rat and goose FAS genes have untranslated exon I sequences (Fig. 2; Refs. 11–13). The untranslated exon I of the human and rat FASs are relatively less conserved, sharing about 65% identity. Interestingly, there is about 90% identity between the human FAS and the rat FAS throughout a region of 170 bp in the 5'-flanking region (Fig. 2), which contains four Sp1 consensus sequences (37) and a consensus sequence of the glucocorticoid response element (38). The putative insulin response sequence overlapping the CAAT box is the binding site of upstream stimulatory factor 1 (9, 17) and is required for sterol response (18, 19). The sequence is also conserved in the goose FAS gene (11). The three FAS sequences even have the same TATA box and CAAT sequences (Fig. 2).

Mapping of Transcription Initiation Sites—To prove the existence of the putative untranslated exon I in human FAS and to map the transcription initiation site(s), primer extension experiments were performed as described under “Experimental Procedures.” The oligonucleotides, oligonucleotide 1 located in exon II, and oligonucleotides 2 and 3, located in the putative exon I (Fig. 3B), were synthesized and used as primers. Based on the location of these oligonucleotides and the consensus sequences of the intron-exon junction, we expected the extension product of oligonucleotide 1 to be 31 nucleotides longer than that of oligonucleotide 2, and the product of oligonucleotide 2 to be 43 nucleotides longer than that of oligonucleotide 3. When total RNA from HepG2 cells was used, the longest
dNAs obtained with oligonucleotides 1, 2, and 3 were 150, 119, and 76 nucleotides, respectively (Fig. 3A, lanes 2, 5, and 8). The lengths of these primer extension products determined the beginning of exon I. In addition, the 150-nucleotide-extended oligonucleotide 1 from exon II confirmed the assignment of the 3'-end of exon I and 5'-end of exon II, which were based on the consensus sequences of the intron-exon junction. When total RNA from T47D cells was used with oligonucleotide 1, however, we could only detect a faint extension product of 150 nucleotides. Moreover, there were significant amounts of new extension products (Fig. 3A, lane 3) that were from 74 to 85 nucleotides in length. These additional extension products were also detected with oligonucleotide 1 in RNA from HepG2 cells (Fig. 3A, lane 2). When oligonucleotides 2 and 3 were used with T47D RNA, no extension products were observed (Fig. 3A, lanes 6 and 9). These results suggested that there may be additional transcription initiation sites located in intron I at positions +1602 and +1613, which are, respectively, 60 and 49 nucleotides upstream of the translation initiation codon ATG (Fig. 2). The ladders in the lanes in Fig. 3 that contain extension products were probably generated by premature termination of reverse transcriptase because of the high GC content.

The results obtained from the primer extension experiments were confirmed by S1 mapping analyses. To map the transcription initiation site at the beginning of exon I, a 115-bp double-stranded DNA fragment was amplified by the polymerase chain reaction (PCR) using AE6 DNA as a template. Oligonucleotide 4 is located in the 5'-flanking region of exon I, and the 32P-labeled oligonucleotide 3 is located in exon I (Fig. 4B). The 115-bp PCR product that contained 39 bp of the 5'-nontranscribed region and 76 bp of the exon I sequence was denatured and hybridized to total RNA from HepG2 cells. S1 mapping analysis was performed as described under "Experimental Procedures." As expected from the results of the primer extension experiments, a 76-bp protected fragment was detected when total RNA from HepG2 cells was used (Fig. 4A, lane 2), thus confirming the assignment of the transcription initiation site Ti I to the beginning of exon I, as shown in Fig. 2.

To confirm the alternative transcription initiation sites present in intron I upstream of exon II, we generated a 300-bp double-stranded S1 probe in the 5'-flanking region of exon II (Fig. 4D). The probe contained 262 bp of intron I and 38 bp of exon II (nucleotides +1393 to +1692). When we used total RNA from T47D cells and from HepG2 cells (Fig. 4C, lanes 1 and 2), we observed three RNA-protected DNA probe fragments that were, respectively, 91, 80, and 38 nucleotides in length. The two longer fragments were generated from RNAs beginning at nucleotides +1602 and +1613, respectively. As predicted (Fig. 4D), these results confirmed those of the primer extension studies described above and indicated the existence of an intron promoter. When the full-length human FAS mRNA that contained exon I hybridized to the DNA probe that did not have the exon I sequence, a 38-nucleotide fragment was generated (Fig. 4, C and D). The relatively low intensity of this band may have resulted from its short-length DNA-RNA hybrid, which did not withstand the S1 digestion. The existence of this third S1 fragment confirmed the 5'-end of exon II, as shown in Fig. 2.

**Deletion Analysis of the Promoter I Region of the Human FAS Gene**—The presence of TATA and CAAT boxes upstream of the Ti I site indicated the presence of a typical eukaryotic promoter in this region. To test the activity of this human FAS promoter, a human FAS promoter-luciferase construct, LC9 (Fig. 1), and its extension and deletion mutants were constructed as described under "Experimental Procedures" and tested in HepG2 cells (Fig. 5). The results from the reporter gene assays of LC9 showed that human FAS promoter I is a relatively strong promoter, for it has about one-third the activity of a CMV promoter-luciferase reporter gene (data not shown). The analysis of various 5' deletions showed that a sequence of about 360 bp upstream of the Ti I site that contain the CAAT and TATA boxes is required for maximum promoter activity under the experimental conditions we used (Fig. 5, LC9-2). This region is highly conserved between the human and rat FASs, which have as much as 90% identity within a region of 170 bp (Fig. 2). Deleting the region between nucleotides −361 and −107 reduced the reporter gene activity to about 53% of that of LC9 (Fig. 5, LC9-4). The deleted region contained putative Sp1
boxes and a consensus sequence of the glucocorticoid response element. However, deleting an additional 20 bp (nucleotides 2107 to 2187) led to a drastic loss of reporter gene activity (Fig. 5, LC9-5), even though the TATA and CAAT boxes were still present. These 20 bp and their 5'-flanking region contain a palindromic sequence, 5' CGGGGCGAAGGGGG 3', whose significance is not known at this time.

Identification of Promoter II Activity in Intron I—To test the existence of an intronic promoter for transcripts initiated at the Ti II (+1602) and Ti III (+1613) sites, a 260-bp human FAS DNA fragment (nucleotides +1394 to +1653) that contained the 5'-end of intron I was cloned upstream of the luciferase gene of vector pXP1 (Fig. 1, LC1). When this reporter gene construct was tested in HepG2 cells, its luciferase activity was 10–20 times higher than that of the promoterless luciferase vector control pXP1 and about 1/15 of that of promoter I construct LC9 (Figs. 5 and 6). These findings confirmed the existence of an intron promoter, termed promoter II, upstream of the Ti II and Ti III sites. Promoter II is a TATA-less promoter and may function through the putative Sp1 boxes that are upstream of the Ti II and Ti III sites.

To identify the DNA region containing the maximum promoter II activity and to study how these promoters work together, we constructed a wild-type luciferase construct (LC3) that contained the 1-kb promoter I region, exon I, and intron I/promoter II (Fig. 1, LC3). The wild-type construct contained both the 5' and 3' intron I splice sites. Surprisingly, its luciferase activity was only 1% of that of promoter I construct LC9 (Figs. 6 and 7). This low level of activity could not have been caused by splicing defects, because sequence analysis showed that the naturally occurring splicing sites of the human FAS gene were reconstructed properly. In the LC3 construct, exon I of the human FAS gene became the 5'-untranslated sequence of luciferase mRNA. This low-level expression of luciferase in the LC3 construct could be due to several factors: 1) the "inhibitory" effect of promoter II, 2) the inhibitory effect of other intron I-specific sequences on the transcription of promoter I, or 3) the influence of the untranslated exon I on the translation.
Human fatty-acid synthase gene promoters were studied by constructing luciferase reporter gene constructs. The human FAS intron I was found to be important in reducing reporter gene expression, even though the length of the intron seemed to be more important. To further confirm the role of the FAS intron I sequence in the inhibition of promoter I activity, deletion analysis was performed. The deletion of human FAS intron I suggested that human FAS promoter I in the wild-type construct LC3 was the only known promoter in these constructs. The fragments containing various lengths of the 5′ regulatory region were released by cutting with HidII and subcloned into pXPl. The relative luciferase activities were shown together with the standard deviations of whatever number (in parentheses) of independent experiments. The values obtained were also represented as the percentage of the reporter gene activity obtained from the LC9 construct, which was arbitrarily designated as 100%.

Promoter II plays a major role in reducing promoter I activity in the wild-type construct LC3. In all the constructs that contained any intron sequences, the intron splice sites were either retained or reconstructed. In construct LC3-55d (Fig. 7), human FAS exon I, which became the 5′-untranslated region of the luciferase mRNA, the reporter gene activity was almost the same as that of promoter I construct LC9, suggesting that human FAS exon I did not significantly affect the luciferase expression.

Both the human and rat FAS genes have very short introns, making intron I one of the longest introns of the human FAS gene. To determine whether negative regulation of the promoter I activity related to intron I is an effect of size or an inhibition specific to the FAS intron I sequence, we substituted human FAS intron I with unrelated introns of similar size. The 1.4-kb human FAS intron I fragment was replaced with either a 1.3-kb intron I fragment of the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (Fig. 8, LC56) or with a 1.4-kb intron II fragment of the human HPRT gene (Fig. 8, LC57). The human HPRT intron genes were introns because they are similar in size to human FAS intron I and do not contain any known promoter or regulatory activity. Since human FAS promoter I is the only known promoter in these constructs that have foreign introns, the reporter gene activities of these constructs were expected to reflect the strength of promoter I. Indeed, their reporter gene activities are about 10 times higher than that of the wild-type construct LC3 (Fig. 8). Although we do not know the splicing efficiency and the influence of the HPRT intron sequences on reporter gene expression, the comparison of the reporter gene constructs containing the HPRT intron with the wild-type construct containing the human FAS intron I suggested that human FAS promoter I in the wild-type construct LC3 is inhibited by specific sequences in human FAS intron I.

Promoter II plays a major role in the inhibition of promoter II activity. Since human FAS intron I contains promoter II activity, we wanted to further delineate the role of the promoter II sequence and the rest of the intron sequence in reducing the expression of promoter II activity. Although the length of the intron seems to play a role in this inhibition, the promoter II sequence seems to be more important (Fig. 7). To further confirm the role of

| Human FAS Gene | CAAT TATA | Exon | RLA | % of LC9 |
|---------------|-----------|------|-----|--------|
| LC11          | LUC       | 1151 | 23,690 ± 2,300 (5) | 87% |
| LC9           | LUC       | 9  | 27,200 ± 2,520 (10) | 100% |
| LC9-1         | LUC       | 9   | 31,580 ± 1,180 (3)  | 116% |
| LC9-2         | LUC       | 9   | 44,200 ± 2,600 (3)  | 160% |
| LC9-3         | LUC       | 9   | 29,250 ± 1,130 (3)  | 106% |
| LC9-4         | LUC       | 9   | 14,690 ± 490 (3)    | 53%  |
| LC9-5         | LUC       | 9   | 2,100 ± 450 (3)     | 6%   |
| pXPl          | LUC       | 9   | 77 ± 10 (10)        | 0.2% |

**Fig. 5.** Deletion analysis of the human FAS 5′-flanking region by using luciferase reporter gene constructs. The construct LC11 was generated by subcloning a 1-kb SstI upstream fragment from AE6 at the SstI site in LC9 (Fig. 2). The 5′-end serial deletions were made from the SstI site in LC9 by using Bal-31 exonuclease as described under “Experimental Procedures.” Fragments containing various lengths of the 5′ regulatory region were released with HindII and subcloned into pXPl at the SmaI and HindII sites. The luciferase gene in pXPl uses its own translation initiation codon, ATG. HepG2 cells were transiently cotransfected with the luciferase (Luc) and showed that promoter I is nonfunctional in the wild-type construct LC3. The maximum promoter I activity did not alter the expression of the reporter gene. As expected, other intron I sequences on promoter I, the deletion of intron I did not change LC3 reporter gene activity. As expected, 5′-end deletion analyses of LC3 showed that the deletion of promoter I did not alter the expression of the reporter gene. Further deletion of intron I sequences, however, caused a 6-fold increase in the luciferase activity. The maximum promoter activity was detected when the 480-bp intron I sequence up-stream of exon II was retained (Fig. 6, LC208). Deletion analysis again confirmed the presence of intron I promoter activity and showed that promoter I is nonfunctional in the wild-type construct LC3 (Fig. 6, LC202 and LC203). In addition, the 5′ intron I sequence (nucleotides +120 to +1182) reduced the expression of promoter II.

**Fig. 6.** The effect of 5′-end serial deletions of the wild-type construct LC3 on reporter gene activity. The construction of reporter gene constructs LC20 and LC3 was as described under “Experimental Procedures” and in the legend of Fig. 1. The 5′-end serial deletions were made from the SstI site in LC3 by using Bal-31 exonuclease as described under “Experimental Procedures.” The fragments containing various lengths of the 5′ regulatory region were released by cutting with HindII and subcloned into pXPl at the SmaI and HindII sites. All other manipulations were carried out as described in the legend of Fig. 5. E = exon; LUC = luciferase; RLA = relative luciferase activities.

| Human FAS Gene | SstI | HindII | ATG | RLA |
|----------------|------|--------|-----|-----|
| LC20           | LUC  | LUC    | LUC | 263 ± 35 (5) |
| LC3            | LUC  | LUC    | LUC | 250 ± 50 (10) |
| LC202          | LUC  | LUC    | LUC | 246 ± 22 (3)  |
| LC203          | LUC  | LUC    | LUC | 390 ± 28 (3)  |
| LC207          | LUC  | LUC    | LUC | 478 ± 50 (3)  |
| LC208          | LUC  | LUC    | LUC | 1,590 ± 85 (3) |
| LC1            | LUC  | LUC    | LUC | 1,028 ± 265 (10) |
| LC3            | LUC  | LUC    | LUC | 174 ± 12 (3)  |
| pXPl           | LUC  | LUC    | LUC | 77 ± 10 (10)  |
provides a major role in the inhibition of promoter I activity. The other transcription initiation sites in the human FAS gene. The Ti I site is mapped at the beginning of exon I and is nine nucleotides upstream of the homologous rat FAST site. It is preceded by a relatively strong promoter that contains TATA and CAAT boxes. An inverted CAAT sequence (ATTG) is also located at 89 to 83 (Fig. 2). The 119-bp untranslated exon is separated from exon II by an intron of 1543 bp. The translation initiation codon ATG is located 8 nucleotides into the 5' untranslated exon; LUC = luciferase; RLA = relative luciferase activities.

**Fig. 8. Substitution of human FAS intron I sequences with human HPRT introns.** A 1.3-kb blunt-ended XhoI-XhoI fragment from intron I of the human HPRT gene or a 1.4-kb KpnI fragment from intron II of the same gene were cloned into the unique KpnI site of the human HPRT introns. A 1.3-kb blunt-ended XhoI-XhoI fragment from intron I of the human HPRT gene or a 1.4-kb KpnI fragment from intron II of the same gene were cloned into the unique KpnI site of the wild-type construct LC3. Thus, the presence of promoter II was responsible for bringing the promoter I activity to a level as low as that of the wild-type construct LC3, regardless of the sequence of the intron upstream. These results clearly suggested that promoter II sequences play a major role in the inhibition of promoter I activity.

In addition, when the reporter gene construct contained both human FAS promoters and a very short intron I sequence that included intron splice sites (Figs. 7 and 8, LC3-16d), its reporter gene activity was still 10 times higher than that of the wild-type construct LC3. When we compared this construct with others that contained both human FAS promoters (Fig. 8, LC3, LC61, and LC62), we were able to attribute the low levels of reporter gene activity obtained either to some sequences present in these introns or to the size of the intron that separates the two FAS promoters. However, as shown in Fig. 7, progressive deletions of intron I while promoter II was retained caused an increase in reporter gene activity, suggesting that a certain distance between the human FAS promoters is needed to reduce the overall reporter gene expression. Apparently, the activity of promoter II and its influence on promoter I expression is dependent on spatial separation of the two promoters, regardless of whether the promoters are separated by human FAS intron I or by HPRT introns.

**Expression of Human FAS Promoter-Luciferase Constructs in Different Human Cell Lines**—The HepG2 cell line was used in most of the experiments described above. Human FAS promoters I- and promoter II-luciferase constructs were also tested in human mammary gland carcinoma T47D cells, human fibroblast HT-1080 cells, and human cervical epithelioid carcinoma HeLa cells to determine the relative strength of these promoter constructs. The test plasmids were cotransfected with CMV-β-gal. The results showed that all the promoter I reporter gene constructs were equally active in all four cell lines (Table I). This finding is expected, since there is no promoter II or other intron sequence in this construct that interferes with the promoter activity. Nevertheless, the promoter II constructs were three to four times more active in HT-1080 and T47D cells than in HepG2 and HeLa cells.

**DISCUSSION**

Primer extension and S1 mapping analyses disclosed three transcription initiation sites in the human FAS gene. The Ti I site is mapped at the beginning of exon I and is nine nucleotides upstream of the homologous rat FAST Ti site. It is preceded by a relatively strong promoter that contains TATA and CAAT boxes. An inverted CAAT sequence (ATTG) is also located at nucleotides −89 to −83 (Fig. 2). The 119-bp untranslated exon I is separated from exon II by an intron of 1543 bp. The translation initiation codon ATG is located 8 nucleotides into the 130-bp exon II. Both the rat and goose FAS genes also have an untranslated first exon (Fig. 2) that are, respectively, 77 and 159 bp in size. These exons have, respectively, 65 and 41% identity to human FAS exon I. Interestingly, there is about 90% identity between the human and rat FAS genes and 61% identity between the human and goose FAS genes throughout a region of 170 bp immediately upstream of the Ti I site of the human FAS gene. These genes even have the same TATA box and CAAT sequences in this region (Fig. 2). Analyzing the deletion of this region clearly indicated that this sequence plays an important role in promoter I activity. The other transcription initiation site of the human FAS gene is mapped at the beginning of the second exon.
Human Fatty-acid Synthase Gene Promoters

The nucleotide sequences common to the 5′-flanking regions of several genes whose transcription increases during adipocyte differentiation have been identified as fat-specific elements (40). The 16-base sequence from nucleotides +1484 to +1499 of the human FAS gene matches the consensus sequence of fat-specific element 1, and the sequence starting from nucleotide +1510 matches the consensus sequence of fat-specific element 2. In addition, there are four Sp1 boxes and a glucocorticoid response element sequence motif in this region. Since the rat FAS sequence in this region (12, 13, 39) has several sequence motifs that are similar to the 3′-end region of the human FAS intron I sequence (Fig. 2), it is quite likely that promoter II also exists in the rat FAS gene. Based on the level of reporter gene expression, this TATA-less promoter II appears to be a weak promoter, having only 4–6% of the activity of promoter I (LC9). However, LC3, a native reporter gene construct that contained promoter I and intron I/promoter II, had only 1% of luciferase activity when compared with that of promoter I construct LC9. Based on the results from the deletion analysis (Fig. 6), it was evident that promoter I is nonfunctional in LC3 and that there are sequences in intron I that reduce the expression of promoter II. This situation probably explains why the activity of the wild-type construct LC3 is lower than that of the promoter II constructs (Figs. 6, LC208 and LC1). In all the constructs in which promoters I and II are both present, the reporter gene activity was very low when compared with that of promoter I (LC9). When promoter I was brought closer to promoter II by deleting most of the intron I sequence (Fig. 7, LC3-16d), the reporter gene activity increased 10-fold. It is difficult, however, to determine whether promoter I or II (or both) is influencing the reporter gene expression in this construct. Based on the results obtained by deletions and substitutions of intron I with heterologous introns (Figs. 7 and 8), it is apparent that promoter II interferes with promoter I function. In addition, spatial separation of these two promoters by an “intrinsic sequence” seems to be important in enhancing this interference. Although we did not anticipate it, the unrelated HPRT introns also reduced the expression of promoter I by 7-fold (Fig. 8, LC56 and LC57), indicating the lack of a specific function of the native intronic sequences excluding promoter II.

Several explanations may be offered for these observations, but we prefer the “roadblock” model as described in Fig. 9. The main principle of this model, based on the data discussed above, is that even though promoter I is very active, the assembly of transcription complexes on promoter II may block the progression of transcription from promoter I and reduce the overall expression. The strength of the promoter II transcriptional assembly could be influenced by the spatial separation between promoters I and II. Another explanation that may be advanced is that there are transcription termination signals or pause sites that are controlled in a tissue-specific manner, as suggested by Proudfoot (47). This proposal assumes that such signals or sites are present in the promoter II region of human FAS. Alternatively, it is also possible that the secondary structure of the untranslated region of the FAS mRNA that contains either exon I or the alternate exon I is responsible for the differential stability and accumulation of these mRNAs (48).

We have not yet characterized the minimal promoter II and intron I sequences that maximally inhibit promoter I transcription. The existence of one promoter I transcript and two promoter II transcripts in FAS mRNA makes exon II and its flanking sequence an alternative exon I. The promoter II activity is probably needed for low-level constitutive expression while the promoter I-mediated transcription is blocked. It is predicted that, under lipogenic conditions, the promoter II activity is somehow minimized to remove the transcriptional block in favor of efficient transcription from promoter I. For example, promoter I transcripts are more prominent in HepG2 cells than in T47D cells, in which promoter II transcripts are predominant (Fig. 3). Analyses of the relative strengths of the promoters in various human tissues are being conducted to further support this view.

Some eukaryotic genes have more than one promoter. Usually, one promoter has a TATA box, and the other does not. For example, the rat ACC gene (41) and the rat CAMP phosphodiesterase gene (42) each have two promoters, one upstream of exon I and the other upstream of exon II. The two promoters of rat CAMP phosphodiesterase are expressed in a tissue-specific manner (42). In rat ACC, promoter I contains TATA and CAAT boxes and is induced under lipogenic conditions, whereas promoter II is a TATA-less intron I promoter (41, 43). Promoter II of rat ACC is expressed constitutively in several tissues (41). Similarily, human ACC has two transcripts that differ in the 5′-flanking region. These transcripts are expressed in a tissue-
specific manner, suggesting the existence of two distinct promoters (44). It is likely that the human FAS gene may have a similar mode of regulation, controlling the abundance of its mRNA in different tissues by alternating the functions of the two promoters.

There are only a few examples of transcriptional interference between RNA polymerase II promoters (45–47). The yeast activator gene intron contains a cryptic promoter that can be activated when the upstream activator gene promoter is deleted (45). In avian leukemia virus, deleting the promoter in the 3′-long terminal repeat sequences can activate the transcription from the 3′-long terminal repeat promoter (46). Artificial tandem constructs of two α-globulin genes were also used to demonstrate the effects of the transcriptional interference of RNA polymerase II transcription (47). In all these cases, however, the upstream promoter transcripts that elongate through the downstream promoter interfere with its function. To the best of our knowledge, we are unaware of any downstream promoter that blocks transcription from the upstream promoter. The physiological significance of the generation of different mRNA species and the transcriptional interference between the two human FAS promoters remains unclear.

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