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Inhibition of the Splicing of Glucose-6-phosphate Dehydrogenase Precursor mRNA by Polyunsaturated Fatty Acids*

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Polyunsaturated fatty acids inhibit the expression of hepatic glucose-6-phosphate dehydrogenase (G6PD) by changes in the amount of G6PD pre-mRNA in the nucleus in the absence of changes in the transcription rate of the gene. We have compared the nuclear accumulation of partially and fully spliced mRNA for G6PD in the livers of mice fed diets high *versus* low in polyunsaturated fat. Consumption of a diet high in polyunsaturated fat decreased the accumulation of partially spliced forms of the G6PD pre-mRNA. Examining the fate of multiple introns within the G6PD primary transcript indicated that in mice fed a high fat diet, G6PD pre-mRNA containing intron 11 accumulated within the nucleus, whereas G6PD mature mRNA abundance was inhibited 50% or more within the same livers. Transient transfection of RNA reporters into primary hepatocyte cultures was used to localize the cis-acting RNA element involved in this regulated splicing. Reporter RNA produced from constructs containing exon 12 were decreased in amount by arachidonic acid. The extent of this decrease paralleled that seen in the expression of the endogenous G6PD mRNA. The presence of both exon 12 and a neighboring intron within the G6PD reporter RNA was essential for regulation by polyunsaturated fatty acid. Inhibition was not dependent on the presence of the G6PD polyadenylation signal and the 3′-untranslated region, but substitution with the SV40 poly(A) signal attenuated the inhibition by arachidonic acid. Thus, exon 12 contains a putative splicing regulatory element involved in the inhibition of G6PD expression by polyunsaturated fat.

Polyunsaturated fatty acids are potent regulators of cellular metabolism. As components of phospholipids, they are involved in signal transduction from specific plasma membrane receptors and are precursors for the synthesis of eicosanoids. When present in the diet of animals or the medium of cells in culture, polyunsaturated fats can both increase and decrease the activity or amount of specific cellular proteins. In this regard, polyunsaturated fatty acids have long been known to decrease the capacity of liver cells to synthesize fatty acids *de novo*.

The pathway of *de novo* fatty acid biosynthesis is essential for the conversion of energy substrates, such as glucose, which are in excess of immediate needs, to fatty acids that can be stored as triacylglycerols. This pathway is most active in liver and adipose tissue and involves a family of enzymes referred to as the lipogenic enzymes (for review, see Ref. 1). These enzymes include ATP-citrate lyase, acetyl-CoA carboxylase, fatty acids synthase, malic enzyme, and glucose-6-phosphate dehydrogenase (G6PD).[1] Consistent with their role in energy metabolism, the activities of these enzymes are induced when animals are fed a high carbohydrate diet and decreased during starvation. Likewise, lipogenic enzyme activity is decreased by a high fat diet, particularly a diet rich in polyunsaturated fatty acids. Regulation of the activities of ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, and malic enzyme involves changes in the rate of transcription of these genes (2–7). In general, these changes in transcription rate fail to account for the magnitude of change in the amount of mRNA for these enzymes. Thus, posttranscriptional regulation has been proposed as an additional mechanism to account for the regulation of the amount of some of these enzymes (6, 8). Regulation of the expression of G6PD is in sharp contrast to other members of the lipogenic enzyme family in that starvation, refeeding, and dietary polyunsaturated fat result in large changes in the amount of G6PD mRNA but do not regulate the transcriptional activity of the G6PD gene (9).

Posttranscriptional regulation of gene expression can occur at multiple steps during RNA processing or by changes in the stability of the mature mRNA. The processing of the nascent transcript to the mature mRNA includes the addition of the 5′-m7GppG cap, splicing, and 3′-end formation. The correct processing of an mRNA is essential for its release from the site of transcription and export to the cytoplasm (10–12). Thus, the efficient and complete maturation of mRNA is a potential control point of gene expression. Previous studies in our laboratory have characterized the posttranscriptional regulation of G6PD by dietary factors. The dietary paradigm of starvation then refeeding causes a 15-fold or more increase in G6PD mRNA abundance. Regulation of G6PD mRNA abundance during the refeeding of starved mice is caused by an increase in the rate of spliced RNA accumulation in the nucleus (13). The increase in spliced RNA accumulation cannot be accounted for by the rate of accumulation of polyadenylated RNA nor does it involve changes in the length of the poly(A) tail. Thus, the G6PD gene is transcribed at a continuous and unchanging rate, and the amount of mature G6PD mRNA produced is regulated during

[1] The abbreviations used are: G6PD, glucose-6-phosphate dehydrogenase; CMV, cytomegalovirus; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; pre-mRNA, precursor mRNA; UTR, untranslated region.

the splicing of the primary transcript to the mature mRNA. The absence of transcriptional regulation of G6PD makes it an ideal model to study this unique posttranscriptional mechanism. We asked if inhibition of G6PD expression by polyunsaturated fatty acids occurs at the same step as positive regulatory stimuli.

In liver, G6PD activity and mRNA amount decrease 80% in mice consuming a diet high in polyunsaturated fat *versus* those fed a low fat diet (9). The decrease in cytoplasmic RNA is preceded by a similar decrease in the amount of mRNA in the nucleus (14). This effect of dietary fat is recapitulated in hepatocytes in primary culture. In this regard, incubation of primary rat hepatocytes with arachidonic acid results in a parallel decrease in the amount of G6PD enzyme activity and mRNA abundance in the absence of a change in the transcriptional activity of the gene (15). Similar to the inhibition by dietary fat, the decrease in G6PD mRNA by arachidonic acid is preceded by a decrease in the amount of mRNA in the nucleus (15). Thus, inhibition of G6PD expression by polyunsaturated fatty acids occurs in the nucleus and is the result of the intracellular actions of fatty acids. Determining the molecular details of this regulation will provide new information on the breadth of mechanisms by which nutrients control gene expression.

In this paper, we present data that define a new mechanism by which polyunsaturated fats inhibit gene expression. We demonstrate that inhibition of G6PD expression involves a decrease in the rate of splicing of the G6PD RNA transcript. Using intact mice, we have identified a slowly spliced intron, intron 11, in mice consuming a high fat diet. We have extended these results using primary rat hepatocytes and have demonstrated that transcripts from pre-mRNA reporters containing exon 12 and a neighboring intron are inhibited by polyunsaturated fatty acids in a manner quantitatively similar to the endogenous gene.

EXPERIMENTAL PROCEDURES

Animal Care and Cell Culture—Male C57BL/6 mice 4 weeks of age were obtained from Charles River Corp. Mice were adapted to reverse cycle room (lights on 7:00 p.m., lights off 7:00 a.m.) for 7 days while maintained on standard chow diet. Mice were switched to a purified basal diet containing 58.43% by weight glucose, 16.45% celuful, 4% salt mix, and 0.02% vitamin mix (Amersham Biosciences) supplemented with 1% (low fat) or 6% (high fat) by weight polyunsaturated fat in the form of safflower oil (Sigma) for 7 days. Mice were sacrificed, and nuclear RNA was isolated at the times indicated in the figures.

Male Sprague-Dawley rats (~200 g from Harlan Laboratories, Indianapolis, IN) fed a standard chow diet (Harlan Teklad) were used for all experiments. Rats were starved for 16 h, refed the high glucose, fat free diet for 8 h, and then starved for 16 h before use as hepatocyte donors. Hepatocytes were isolated by a modification of the technique of Seglen (16) as previously described (15). Hepatocytes (3.3×10^6) were placed in 60-mm dishes coated with rat tail collagen in Hi/Wo/Ba medium (Waymouth's MB752/1 plus 20 mM HEPES, pH 7.4, 0.5 mM serine, 0.5 mM alanine, 0.2% bovine serum albumin) plus 5% newborn calf serum (37 °C, 5% CO_2). Cell viability in all experiments was 90% or greater as estimated by trypan blue (0.04%) exclusion. After 3–4 h, the medium was replaced with serum-free medium. After an additional 16 h of incubation, the medium was replaced with medium containing the treatments indicated in the figure legends and a Matrigel overlay (0.3 mg/ml; BD PharMingen) (17). Subsequently, the medium was changed every 12–24 h to one of the same composition but without Matrigel. Arachidonic acid (Nu-Check Prep, West Elysian, MN) was bound to bovine serum albumin (18). The fatty acid (4 mM), albumin (1 mM) stocks contained butylated hydroxytoluene (0.01%), and the medium contained α-tocopherol phosphate, disodium (10 μg/liter), to minimize oxidation of fatty acids.

Transfection was done using Lipofectin following the manufacturer's protocol (Invitrogen) and using 2–4 μg of test DNA and 1 μg of RSV CAT to control for transfection efficiency. The ratio of DNA to liposome reagent was 1:6.7 in all experiments. Transfection was begun after 4 h of culture, and the transfection medium remained on the hepatocytes

for 16 h. In transfection experiments the Matrigel overlay was added 4 h after the transfection began (17).

Constructs for Transfection—Plasmids used in the analysis of cis-acting elements in the G6PD primary transcript were made using pGL3-Basic (Promega Corp., Madison, WI) as the vector backbone. The luciferase gene was removed from this vector, and portions of G6PD genomic DNA or the G6PD cDNA were inserted in its place. Either the CMV promoter/enhancer sequences (1–640 bp from the pCMVβ vector, CLONTECH) or the G6PD promoter sequences (−780 to +3) were inserted into the multiple cloning site to drive expression of the test sequences. The plasmids contained either the SV40 polyadenylation signal provided in the plasmid or the G6PD polyadenylation signal. The RSV CAT plasmid used to control for transfection efficiency was constructed using the pCAT3 Basic vector (Promega) and inserting the RSV long terminal repeat from pRSV CAT (19).

Isolation of Total RNA and Northern Analysis—Total RNA from 2–3 plates per treatment was isolated by the method of Chomczynski and Sacchi (20). Quantitation of RNA using Northern analysis was done as previously described (9).

Isolation of Nuclear RNA—Nuclei from liver were isolated by a modification (13) of the method of Leppard and Shenk (21). This protocol results in the isolation of nuclear RNA that is in the processing pathway and is essentially devoid of cytoplasmic contamination (13). In later experiments using probes with two introns (Figs. 3 and 4), the DNase I digestion and high salt extraction steps were eliminated because these probes permit the detection of pre-mRNA at two different steps in splicing. The absence of cytoplasmic contamination in this fraction was assessed as described (13). Cytoplasmic RNA in the nuclear RNA preparations was less than 1%.

Probe Design and Ribonuclease Protection Assay—The following probes were designed for use in the ribonuclease protection assay (Fig. 1). The exon 2-intron 2 (E2-I2) and exon 8-intron 8-exon 9-intron 9 (pBG2) probes have been previously described (13). Probes to intron 6-exon 7-intron 7-exon 8 (pBG1) and exon 10-intron 11 (p200) were constructed by subcloning G6PD genomic DNA into pBluescript KS+ (Stratagene, La Jolla, CA). An additional probe, pJW1, was designed that hybridized to exon 10-intron 10-exon 11-intron 11 and exon 12. The template for this probe was synthesized from a genomic subclone by PCR amplification. The 5′ primer was 5′-CGGAATTCAACTTATG-GCAACAG-3′; the underlined sequence is an *Eco*RI site for subcloning followed by G6PD exon 10 sequence. The 3′ primer was 5′-AAGGAT-TCCTCTCGATCAATCTTG-3′; the underlined sequence is a *Bam*HI site for subcloning followed by G6PD exon 12 sequence. After amplification, the DNA was subcloned into pBluescript KS+, and the authenticity of these sequences was verified by sequencing.

Two templates were designed for use in ribonuclease protection assays with RNA from transfected rat hepatocytes. Rat and mouse G6PD exon 13 templates were synthesized by PCR amplification of genomic DNA. These probes were targeted to a region of exon 13 that contains substantial mismatch between the rat and mouse sequences. In this way the rat probe detects mRNA produced from the endogenous G6PD gene but not the transfected gene, whereas the mouse probe detects mRNA produced from the transfected DNA and not the endogenous gene. The primer pairs for the rat template were 5′-CGGAATTCTG-GAGGGACAATGACCAAACC-3′ and 5′-CGCGGATTCCGGTTGGAT-GAGGCTGACTATGGAC-3′, where the single and double underline represent *Eco*RI and *Bam*HI restriction sites, respectively. The fragment was subcloned into pBluescript KS+ and sequenced. The mouse exon 13 template was as previously described (14). Templates for probes to rat β-actin, CAT, and 18 S were purchased from Ambion, Inc. (Austin, TX). All templates were linearized before use in the *in vitro* transcription reaction.

Antisense RNA probes were synthesized in an *in vitro* transcription reaction. The probe and RNA were hybridized at 45 °C overnight, and RNase digestion was as previously described (14). The resulting hybridization products protected from RNase digestion were separated in a 5% denaturing polyacrylamide gel. The gel was dried and placed in a storage phosphor cassette for 1–3 days. Images were quantified using ImageQuant software by Molecular Dynamics (Amersham Biosciences). In Figs. 2–4, the units obtained from the image analysis were expressed per number of cytosines in the protected fragment to permit comparison of the amount of RNA between fragments of different size.

RNA from hepatocytes that had undergone transient transfection frequently contained significant amounts of residual plasmid DNA. Because this DNA would also bind to probes and result in protected fragments of the same size as the target RNA, the contaminating DNA was eliminated by digestion of the RNA with RNase-free DNase 1 (10 units/10-μg aliquot of hepatocyte RNA in 10 mM Tris, pH 7.5, 25 mM

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MgCl$_2$, 5 mM CaCl$_2$) for 30 min at 37 °C before hybridization with the radioactive probes.

RESULTS

Inhibition of the Accumulation of G6PD-spliced mRNA by Dietary Polyunsaturated Fat—To investigate if dietary polyunsaturated fatty acids inhibit the efficiency of splicing of the G6PD transcript, we compared the amount of unspliced and spliced G6PD mRNA in the livers of mice fed the low fat *versus* the high fat diet. The amount of hepatic G6PD mRNA varies in amount in response to the normal feeding behavior of the mouse. Mice eat during the dark cycle and are relatively inactive during the light cycle. At the start of the dark cycle, the level of G6PD mRNA is very low. As the mice consume the low fat diet, the amount of G6PD mRNA increases 7-fold or more (14); this increase occurs after a lag of 2–4 h. As the light cycle begins, the amount of G6PD mRNA decreases, returning to a very low level. Consumption of a diet high in polyunsaturated fat results in a 50% decrease in the amount of hepatic G6PD mRNA at the beginning of the dark cycle, and an attenuation of the feeding induced increase to less than 2-fold (14). We reasoned that the continued increase in G6PD mRNA in the livers of mice fed a high fat diet reflected the stimulation in gene expression due to the carbohydrate in the diet. Thus, to measure only gene expression effects due to dietary fat, we measured G6PD mRNA abundance during the first 4 h of the feeding cycle, a time before the major increase in mRNA accumulation. RNA was isolated from both the cytoplasm and the nuclear insoluble fractions of mouse liver after the animals were adapted to either a low fat or a high fat diet for 7 days. The nuclear insoluble fraction is enriched in nascent RNA being transcribed and undergoing RNA processing (22–24). Two probes separated by 12 kilobases were used to detect G6PD RNA (Fig. 1, *E2-I2* and *p200* probes). Each probe hybridized across an exon/intron junction and, thus, measured G6PD RNA that contained that intron and RNA from which that intron had been spliced. The protected fragments are referred to as unspliced and spliced RNA, respectively, even though both protected fragments represent a mix of RNA containing one or more of the G6PD 12 introns.

Feeding mice a high fat diet resulted in a 60–70% decrease in the amount of spliced G6PD RNA in both the nuclear and cytoplasmic fractions of mouse liver (Fig. 2, 0 h time point). Between 0 and 2 h of feeding, the amount of unspliced RNA (E2-I2 protected fragment) in the nucleus remained very low and was not different between mice fed the high fat and the low fat diet. This lack of difference in the amount of pre-mRNA is consistent with our previous data demonstrating that transcription of the G6PD gene is not regulated by polyunsaturated fat (9). By 4 h, the amount of G6PD unspliced mRNA had increased in mice fed the low fat diet, whereas in mice fed the high fat diet, the amount of unspliced mRNA remained at a low basal level. At all time points, the amount of spliced RNA (E2 protected fragment) in the nucleus was 5–10-fold greater than the amount of unspliced RNA; however, this increase in the amount of spliced RNA was attenuated at all time points by the high fat diet. The changes in the amount of spliced RNA in the nucleus were similar to the changes in the amount of mature mRNA in the cytoplasm. Furthermore, the specific activity (phosphorimaging units/number of cytosines) of the spliced RNA was the same in both the cytoplasmic and nuclear pools, consistent with our previous results demonstrating that G6PD regulation by polyunsaturated fatty acids occurs in the nucleus (14). Similar results were obtained with a probe (p200) that hybridizes to the exon 10-intron 10 splice junction of the G6PD RNA (data not shown).
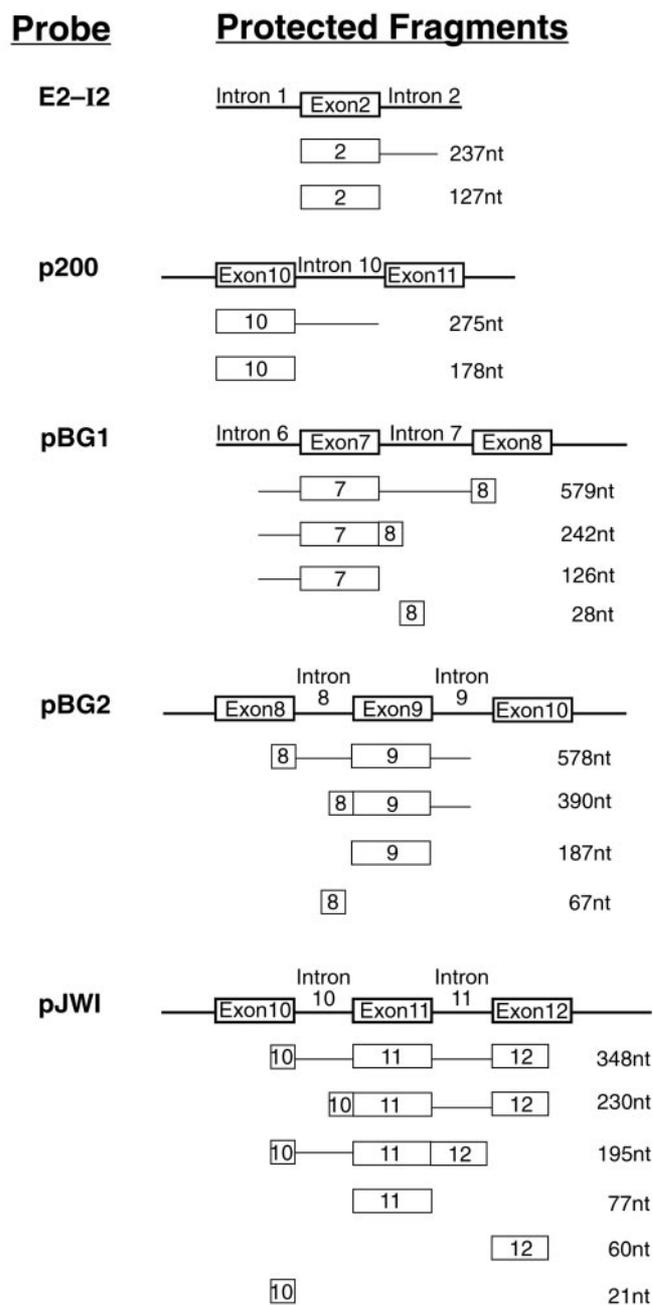
The decrease in the amount of spliced RNA in the nucleus

FIG. 1. **Probes and protected fragments used in ribonuclease protection assays with mouse RNA.** The *lines* and *boxes* schematically depict the structure of the pre-mRNA. To the *left* of the schematic are the working names for each of the probes. Depicted beneath the pre-mRNA structure are the protected fragments that are detected with each probe. The largest protected fragment in each group identifies pre-mRNA that is "unspliced." Protected fragments retaining one of the two introns are referred to as "partially spliced" RNA. "Spliced" RNA is quantified using the protected fragments representing only the exon sequences. The riboprobes used to detect these RNA species were 41–90 nucleotides larger than the full-length protected fragment, so that undigested probe could be discriminated from largest protected fragment.

could reflect a decrease in the rate of splicing of the primary transcript or a decrease in the stability of the fully spliced transcript. To discriminate between these possibilities, the amount of RNA early in the splicing process was measured using RNase protection assays and a probe that hybridized across two introns (exon 8, intron 8, exon 9, and intron 9, pBG2). Four protected fragments were detected representing G6PD pre-mRNA that contained both introns (unspliced),
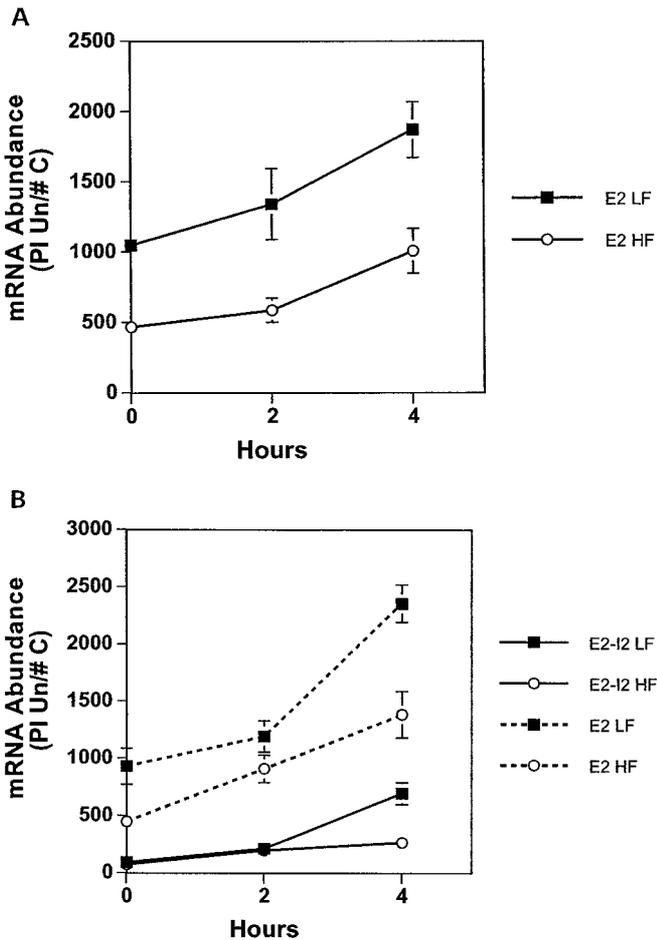
A

B

FIG. 2. **Dietary fat inhibits the accumulation of spliced RNA for G6PD.** RNA was isolated from the cytoplasm (*A*) and the nuclear insoluble (*B*) fractions of mouse liver after adaptation to a high fat or low fat diet. Zero hour represents the start of the feeding (dark) cycle. The isolated RNA (20 μg) was analyzed by RNase protection assay using the exon 2-intron 2, depicted in Fig. 1. The amounts of the unspliced (exon 2-intron 2, E2-I2) and spliced (exon 2, E2) RNase protection products were imaged and quantified using phosphorimaging and ImageQuant software. Each point represents the mean ± S.E. (*n* = 4 mice). The values are the ImageQuant units normalized for the C content of the protected fragment (*PI Un/# C*). Each RNase protection assay also included a probe for 18 S rRNA to detect any loss of RNA during the assay or error in quantitation. No difference was observed in the amount of 18 S RNA in each sample. The significance of the differences between G6PD E2 mRNA abundance in the high fat *versus* the low fat nuclear samples was analyzed using analysis of variance. Dietary fat caused a significant decrease in spliced mRNA abundance in the nucleus (*p* < 0.0001). Identical results were obtained in a separate experiment using the p200 probe (Fig. 1) for the exon 10-intron 10 region of the pre-mRNA (data not shown).

A

B

Hour	Low Fat	High Fat
	(partially-spliced/unspliced)	
0	1.86 ± 0.3	0.42 ± 0.2
2	1.80 ± 0.1	1.21 ± 0.2
4	2.54 ± 0.1	1.42 ± 0.1

C

FIG. 3. **Inhibition of G6PD mRNA abundance by dietary fat occurs early in splicing.** RNA was isolated from the nucleus of mouse liver after adaptation to the high fat or low fat diet. Zero hour represents the start of the feeding (dark) cycle. *A*, the isolated RNA (30 μg) was analyzed by RNase protection assay using the pBG2 probe, depicted in Fig. 1. The amounts of unspliced (exon 8-intron 8-intron 9-intron 9) and partially spliced (exon 8-exon 9-intron 9) RNase protection products were imaged and quantified using phosphorimaging and ImageQuant software. Each point represents the mean ± S.E. (*n* = 3 mice). The values are the ImageQuant units normalized for the C content of the protected fragment (*PI Un/# C*). These data are representative of two independent experiments, which showed similar results. *B*, the amounts of the partially spliced and unspliced RNA were quantified for each separate mouse at each time point in the experiment in *A*. The values were normalized for C content, and the ratio of partially spliced/unspliced RNA was calculated. Each value is the mean ± S.E. of *n* = 3 mice. *C*, the nuclear RNA was analyzed using RNase protection assays, and the pBG1 probe is depicted in Fig. 1. The amount of partially spliced RNA (intron 6-exon 7-exon 8) was quantified and expressed as described above. *HF*, high fat; *LF*, low fat.

pre-mRNA that had only intron eight spliced (partially spliced), and two fragments representing G6PD RNA that had both introns removed (fully spliced). Protected fragments corresponding to spliced introns were not detected. At 0 h, the amount of unspliced RNA was similar in mice fed both the low fat and high fat diets despite a 50% decrease in the amount of fully spliced RNA (Fig. 3*A*, *overlapping circle* and *square*, and data not shown). The amount of partially spliced RNA was greater at all time points than the amount of unspliced RNA. As the mice consumed the low fat diet, the amount of partially spliced RNA increased 7–8-fold, whereas only a 2–3-fold increase in partially spliced RNA was observed in mice fed the high fat diet. The attenuation of partially spliced mRNA accumulation with the high fat diet resulted in a decrease in the ratio of partially spliced to unspliced RNA between the two
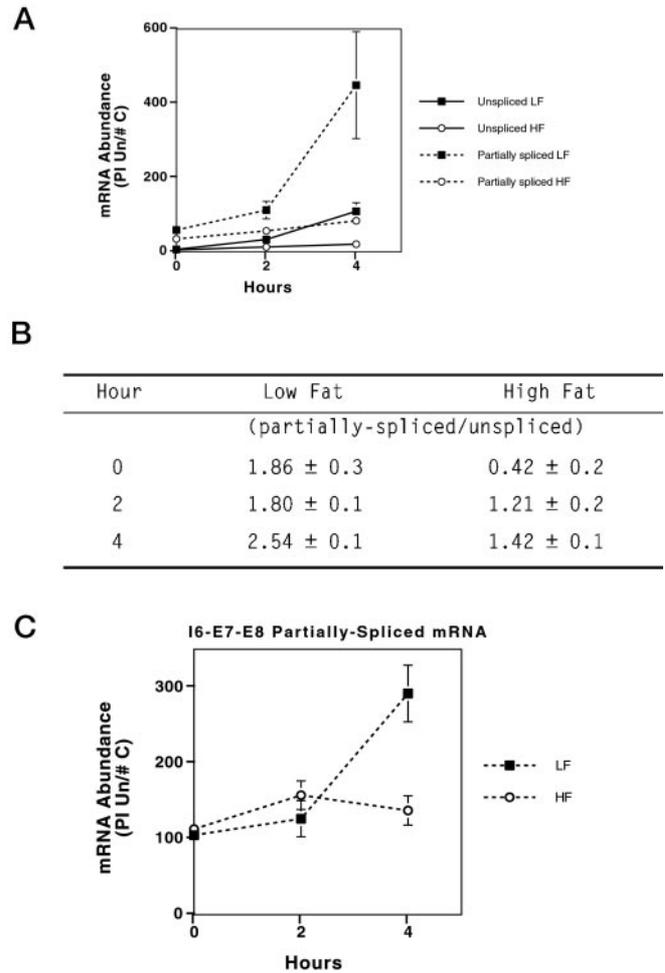
dietary groups (Fig. 3*B*). Thus, dietary polyunsaturated fat caused a decrease in the accumulation of partially spliced RNA. These data are consistent with regulation at an early step in RNA processing rather than a change in stability of the mature mRNA in the nucleus.

A decrease in the ratio of the amount of partially spliced to unspliced RNA in mice fed the high fat diet compared with the low fat diet could indicate that the splicing reaction itself is inhibited by dietary polyunsaturated fatty acids. In such a case the improperly spliced RNA would be targeted for degradation in the nucleus (11, 12). Thus, we examined other intron-exon boundaries to determine whether any intron was selectively retained in the G6PD transcript when mice were consuming a high fat diet. A probe to intron 6, exon 7, intron 7, and exon 8

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(pBG1) detected RNA with both introns present, RNA from which intron only 7 has been spliced and RNA from which both introns are removed. Partially spliced RNA representing retention of intron 6 increased 3-fold in mice fed the low fat diet (Fig. 3*C*). Consumption of the high fat diet attenuated the accumulation of this partially spliced RNA, consistent with the previous results.

A distinctly different result was obtained when a probe was used to the exon 10, intron 10, exon 11, intron 11, and exon 12 (pJW1) region of the pre-mRNA. Use of this probe with nuclear RNA resulted in protected fragments representing RNA containing both intron 10 and 11, RNA from which intron 10 had been spliced, RNA from which intron 11 had been spliced, and RNA from which both introns had been removed. Thus two partially spliced RNA intermediates were detected in contrast to the results with other probes where the partially spliced RNA consistently contained only one of the two introns represented in the probe (Fig. 4*A*). Even more striking was the increase in abundance of partially spliced RNA containing intron 11 in mice fed the high fat diet (Fig. 4*B*, *NoI/O HF bars*). The increase in abundance of this partially spliced RNA occurred despite a decrease of 61% or more in fully spliced RNA detected in the nucleus (Fig. 4*A*, exon 11 and 12 bands, and data not shown for the other time points). Likewise, the amount of unspliced RNA increased with time in mice fed the low fat diet, and this increase was inhibited in mice fed the high fat diet. This result was reproducible both between mice within a single experiment and between different experiments with different groups of mice. In the mice fed the high fat diet, the pre-mRNA retaining intron 11 must ultimately be degraded since the amount of fully spliced RNA (protected fragments representing exons 11 or 12) was decreased within the same aliquot of RNA (Fig. 4*A*). These results are consistent with a decrease in the efficiency of pre-mRNA splicing in mice fed a high fat diet.

Inhibition of G6PD pre-mRNA Reporter Expression in Primary Rat Hepatocytes—Incubation of rat hepatocytes in primary culture with arachidonic acid results in a decrease in the expression of the G6PD gene similar to the decrease observed in intact animals due to dietary polyunsaturated fatty acids. This inhibition is caused by a decrease in the amount of G6PD pre-mRNA in the nucleus in the absence of changes in the transcriptional activity of the gene (15). We used primary hepatocytes to determine whether arachidonic acid would inhibit the amount of RNA expressed from pre-mRNA reporter constructs expressing G6PD RNA. Each pre-mRNA reporter contained the CMV promoter. Included in the transfections was a reporter plasmid containing the RSV promoter and the CAT gene, which served as an internal control for transfection efficiency. In general, variation in CAT expression was less than 10% between treatments (data not shown). To determine that the activity of the CMV promoter was not regulated by arachidonic acid, rat hepatocytes were transfected with CMV β-galactosidase and incubated with and without arachidonic acid. Incubation of the hepatocytes with arachidonic acid did not regulate either the amount of β-galactosidase mRNA (Table I) or enzyme activity (data not shown). Likewise, the amount of CAT mRNA was the same between treatments, indicating that transfection efficiency was similar with and without arachidonic acid. The 40–70% decreases in the amount of endogenous G6PD mRNA indicated that the hepatocytes were responsive to arachidonic acid. Thus, the activity of the CMV promoter was not regulated by arachidonic acid.

The G6PD gene is 18 kilobases long and contains 13 exons. To test for cis-acting elements in the RNA, plasmids were constructed containing different pre-mRNA reporters repre-
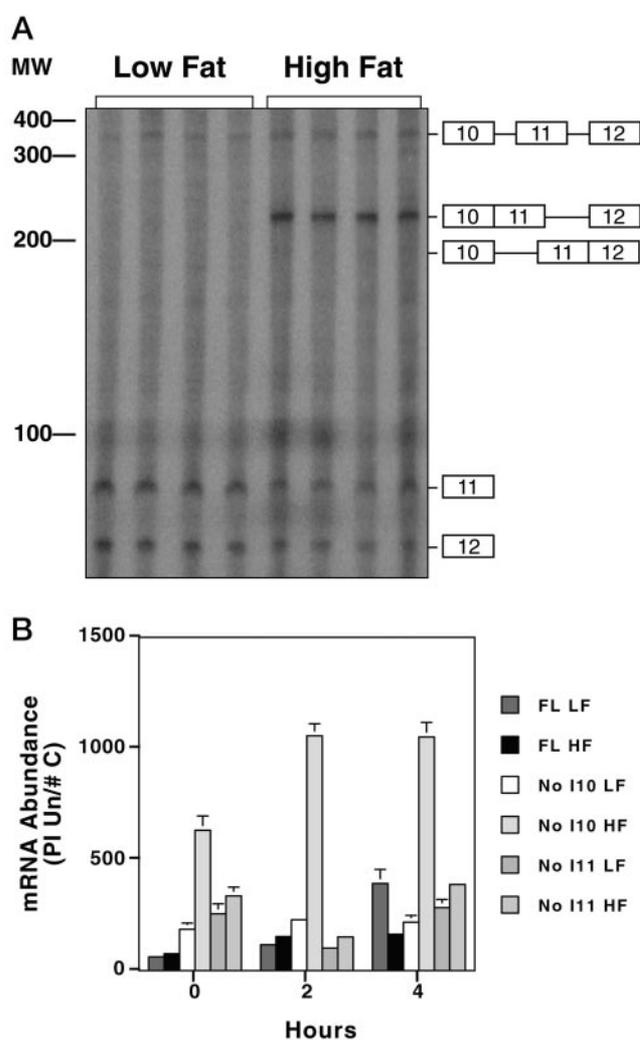

FIG. 4. **Pre-mRNA containing intron 11 selectively accumulates in mice fed the high fat diet.** RNA isolation and the dietary protocol were as described in Fig. 3. The isolated RNA (30 μg) was analyzed by RNase protection assay using the pJW1 probe, depicted in Fig. 1. *A*, a representative assay is shown. The size and structure of the protected fragments are shown on the *right* of the figure. *Single lines* represent introns, and *rectangles* represent exons. Each *lane* represents RNA from a single mouse from the 2-h time point shown in *B*. This assay is representative of two separate experiments and nuclear RNA preparations. *B*, RNase protection products representing unspliced and partially spliced RNA were quantified using ImageQuant software. Each *bar* represents the mean ± S.E. of $n = 4$ mice with the exception of the 4-h low fat bars, which are $n = 3$ mice. The phosphorimaging units were normalized for the C content of the fragment (*PI Un/# C*). The identity of the *bars* is listed on the right of the figure. *HF*, high fat; *LF*, low fat; *FL*, full-length protected fragment.

senting subsets of the 18-kilobase gene (Fig. 5). An additional mRNA reporter was constructed that contained the full-length cDNA for G6PD. The genomic DNA and the cDNA were both of murine origin and were transfected into rat hepatocytes. Exon 13 sequences differ sufficiently between rats and mice such that RNase protection assays and a riboprobe to this region could be used to distinguish between transcripts produced from the pre-mRNA reporter plasmid and from the endogenous mRNA (Fig. 6). In all transfected cells, the amount of endogenous G6PD mRNA was decreased 40% or more due to incubation with arachidonic acid. Expression of β-actin mRNA was not regulated, indicating that changes in G6PD mRNA accumulation in the hepatocytes was not due to a generalized effect of arachidonic acid on gene expression.

A pre-mRNA reporter containing DNA between intron 6 and

TABLE I

The activity of the CMV promoter is not regulated by arachidonic acid in rat hepatocytes

Experiment	Treatment	β-Galactosidase	CAT	G6PD
1	G + I	30.5 ± 4	75.4 ± 23	12.2 ± 2
	G + I + FA	28.9 ± 4	69.3 ± 18	2.9 ± 0.5
2	G + I	82.7 ± 8	382.5 ± 5	32.6
	G + I + FA	77.4 ± 11	413.5 ± 22	18.9
3	G + I	82.5 ± 2	25.9 ± 1	72.5
	G + I + FA	75.9 ± 1	30.9 ± 2	26.7

Isolated hepatocytes were cotransfected with pCMVβ and pRSV -CAT in experiments 1 and 2 and with pCMV-GL3-gal and pRSV -CAT in experiment 3. Total RNA was isolated after 24 h with the indicated treatments, and the amounts of β-galactosidase, CAT, and G6PD mRNA were measured. The values are the mean $\pm$ S.E. for $n = 3$ plates; the actual numbers from ImageQuanT were multiplied by 10^{-4} for ease of reporting. The absence of a S.E. indicates only $n = 2$ plates were assayed. *G,* glucose (25 mM); *I,* insulin (0.1 μM); FA, arachidonic acid (250 μM).
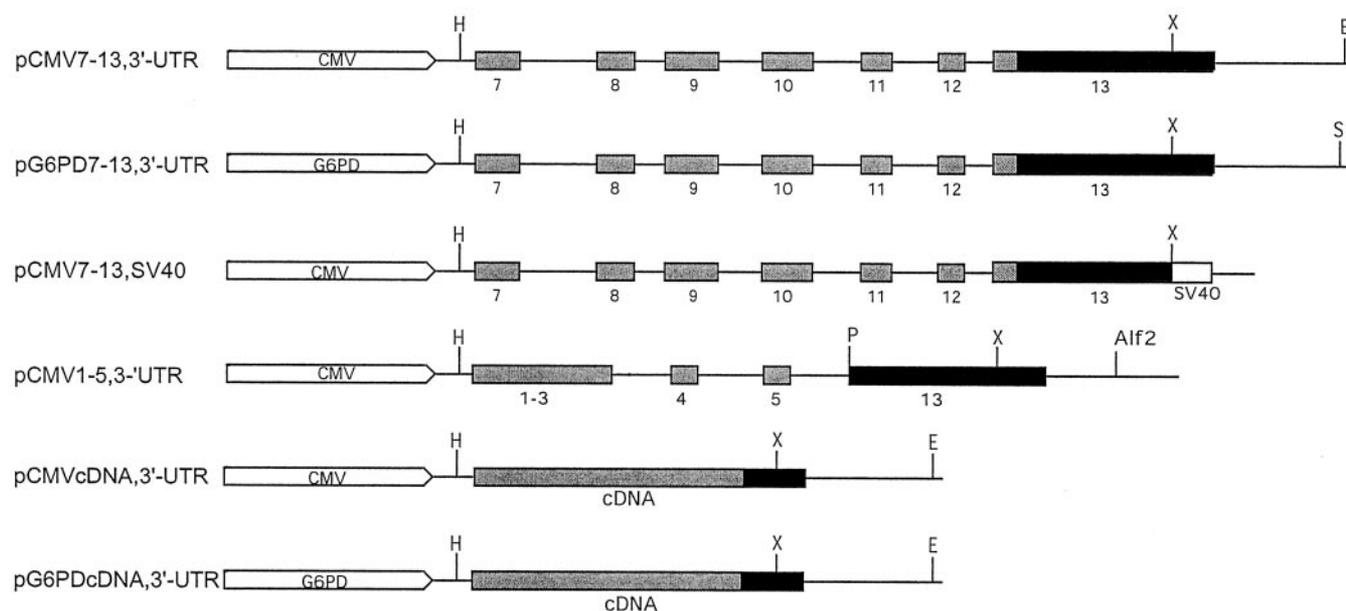
G6PD Pre-mRNA Reporters

FIG. 5. **Structure of the G6PD pre-mRNA reporters used in transfection assays.** Portions of G6PD genomic DNA or the cDNA were subcloned into the pGL3-Basic vector as described under "Experimental Procedures." The DNA was of mouse origin. Either the CMV promoter or the G6PD promoter drove transcription of the reporter RNA. The *Hin*dIII (*H*) site represents the start of genomic DNA in the plasmids containing exons 7–13. In the remaining plasmids this site represents regions of the G6PD 5′-flanking DNA beginning at base −9. The *Eco*R1 (*E*), *Sma*I (*S*), and *Alf*2 sites are present in the genomic DNA 3′-end of the G6PD gene and represent the 3′-end of the inserted DNA. *Gray-shaded rectangles* represent G6PD exons. The *black-shaded* portions of the *rectangles* represent the portion of exon 13. which is the 3′-UTR. The *Xba*I (*X*) site is 136 nucleotides from the 3′-end of exon 13. The sizes in the figure are approximate.

the 3′-end of the gene was robustly expressed in rat hepatocytes incubated with insulin and glucose (Fig. 6). This plasmid contains exon 11, intron 11, exon 12, and intron 12 the region implicated in the decreased splicing efficiency by the high fat diet in mouse liver. Incubation of the rat hepatocytes with arachidonic acid resulted in a 51% decrease in the level of this reporter transcript. Expression of the CAT mRNA was similar between the treatments; thus, the decrease was not a consequence of differences in transfection efficiency or a generalized effect on expression of transfected plasmids. The decrease in the reporter transcript expression was similar to the decrease in the amount of rat G6PD mRNA, indicating that the regulation of reporter transcript expression mirrored the regulation of the endogenous G6PD gene.

Regulated splicing can involve interactions between splicing regulatory elements within the regulated exon or intron and regulatory elements in the promoter or 3′-UTR. We next tested if the regulated expression of this reporter pre-mRNA is retained in the context of a different promoter or polyadenylation signal. A derivative of the original plasmid reporter (pCMV7–13, 3′-UTR) was made in which the CMV promoter was replaced with the G6PD promoter (pG6PD7–13, 3′-UTR). The amount of reporter

RNA was decreased by arachidonic acid when the G6PD promoter drove production of the reporter RNA (Fig. 7). This confirmed that the CMV promoter was not causing the arachidonic acid-induced decrease in mRNA amount. A second derivative involved replacement of 137 nucleotides of the 3′-UTR and the entire downstream genomic DNA with the SV40 polyadenylation signal (pCMV7–13, SV40). When the amount of reporter transcript was measured in hepatocytes transfected with this plasmid (pCMV7–13, SV40), the inhibition by arachidonic acid was attenuated (Fig. 7). However, the amount of the reporter transcript was decreased 14.5%, suggesting that this region of the G6PD 3′-UTR may augment the regulation but was not required for inhibition by arachidonic acid.

It is possible that the presence of any G6PD intron could be involved in the inhibitory effect of arachidonic acid on the expression of these reporter RNAs. To test this, a pre-mRNA reporter was constructed that contained introns 3 and 4 and part of 5 within the context of G6PD cDNA sequences (Fig. 5; pCMV1–5, 3′-UTR). After transfection of this reporter into the rat hepatocytes, the transcripts produced were measured using the RNase protection assay and reporter-specific probe. Incubation with arachidonic acid did not regulate the level of these
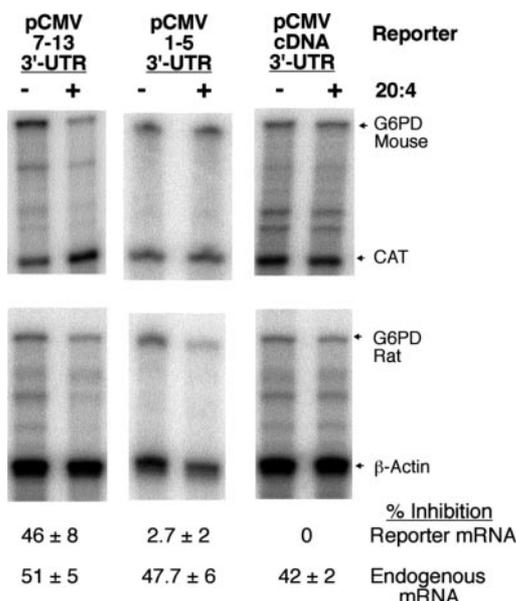
FIG. 6. **Arachidonic acid (20:4) inhibits the accumulation of G6PD reporter transcripts.** Primary rat hepatocytes were transfected by incubation with liposomes and the G6PD pre-mRNA reporter constructs depicted in Fig. 5 plus RSV CAT as a control for transfection efficiency. After 14 h of incubation with the DNA-liposome complexes, the medium was changed to one of the same composition but containing glucose (25 mM) and insulin (0.1 μM) with or without albumin-bound arachidonic acid (175 μM). The hepatocytes were incubated for 24 h with the treatments, and then total RNA was isolated from three pooled dishes (60 mm) of hepatocytes. The amounts of reporter transcript and endogenous mRNA were measured using RNase protection assays and transcript specific riboprobes. G6PD mouse represents transcripts from the pre-mRNA reporter; CAT represents transcripts from RSV CAT; G6PD rat and β-actin represent endogenous rat mRNA. The results of representative experiments are shown. The values beneath the figure are the percent inhibition due to arachidonic acid and are the mean ± S.E. of separate experiments representing different rat hepatocyte isolations. The amount of G6PD mouse transcript was divided by the amount of CAT transcript ($\times100$) to correct for differences in transfection efficiency. The G6PD rat signal is divided by the β-actin signal ($\times100$) to correct for differences in RNA loading. Repetition was as follows: pCMV7–13, 3′-UTR ($n = 7$); pCMV1–5, 3′-UTR ($n = 3$), and pCMVcDNA, 3′-UTR ($n = 3$).

reporter transcripts but did decrease endogenous G6PD expression by 60% (Fig. 6). Thus, regulation of G6PD expression requires RNA sequences between intron 6 and the end of the gene and not merely the splicing of G6PD introns. A similar result was observed when the expression of an RNA reporter containing only the cDNA sequences were measured (Fig. 6; *pCMVcDNA*). Substitution of the G6PD promoter for the CMV promoter failed to reconstitute the inhibition (Fig. 7; pG6PDcDNA). These results indicate that inhibition of G6PD mRNA accumulation by arachidonic acid requires introns that are 3′ of intron 6. This suggests that the functional assay in rat hepatocytes recapitulates the regulated splicing observed in intact mice fed a high fat diet.

To further localize the RNA element involved in inhibition of G6PD expression by arachidonic acid, a series of deletion constructs were made using unique restriction sites in this region of the genomic DNA. Pre-mRNA reporters containing successively smaller regions of the G6PD transcript were constructed and transfected into primary rat hepatocytes (Fig. 8). All constructs contain the same 91 nucleotides downstream of the TATA box in the CMV promoter, which were also present in the previous reporter constructs. RNA from the reporter constructs was readily detected in the RNase protection assays. The amount of reporter RNA was decreased by incubation with arachidonic acid in all constructs that contained sequences
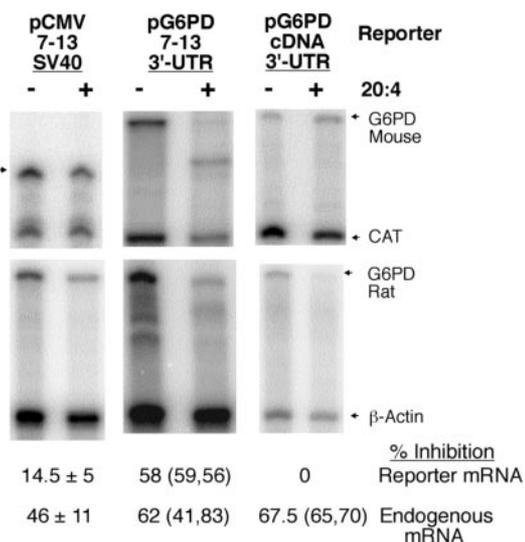

FIG. 7. **Inhibition of G6PD reporter RNA expression does not require the G6PD promoter or 3′-UTR.** Primary rat hepatocytes were transfected, and the reporter RNA was analyzed as described in the legend to Fig. 6. *G6PD mouse* represents mRNA produced from the RNA reporters, and *G6PD rat* represents mRNA produced from the endogenous gene. The protected fragment resulting from RNA produced from pCMV7–13, SV40 is smaller than the reporters containing the G6PD 3′-UTR and is indicated by the *arrow* on the *left*. The *band* in the *pG6PD7–13, 3′-UTR, +20:4* lane is incompletely digested CAT probe. The *values beneath* the *figure* are the percent inhibition due to arachidonic acid and are the mean ± S.E. of $n = 4$ separate experiments in the case of pCMV7–13, SV40, or the average of $n = 2$ separate experiments in the case of pG6PD7–13, 3′-UTR and pG6PDcDNA, 3′-UTR. The *values* in *parentheses* are the values for the individual experiments. The amount of G6PD mouse transcript was divided by the amount of CAT transcript ($\times100$) to correct for differences in transfection efficiency. The G6PD rat signal is divided by the β-actin signal ($\times100$) to correct for differences in RNA loading.

between the start of exon 12 through the 3′-end of the gene (Fig. 8). The extent of this inhibition was similar to the inhibition of the endogenous gene. Furthermore, this region of the gene was able to confer inhibition by arachidonic acid onto a heterologous RNA, β-galactosidase (*pβ-gal ex12–13*) and to a portion of the G6PD cDNA (*pcDNA ex11–13*; Fig. 8). The pβ-gal ex12–13 construct contained only the last 60 nucleotides of exon 12, further localizing this element in the exon. In all experiments the expression of the endogenous β-actin mRNA was not regulated by arachidonic acid, indicating that arachidonic acid did not have a generalized inhibitory effect on cellular metabolism (data not shown). Deletion of exon 12 from the reporter construct abrogated the inhibition by arachidonic acid despite a robust inhibition of the endogenous gene (pCMV in12–13). Thus, a cis-acting RNA element involved in inhibition of G6PD RNA accumulation by arachidonic acid localizes to exon 12.

DISCUSSION

Dietary polyunsaturated fatty acids are negative regulators of hepatic lipogenesis that exert their effects both at transcriptional (for review, see Ref. 1) and posttranscriptional (9, 14, 25, 26) steps. The present study is the first to demonstrate that polyunsaturated fat can inhibit gene expression by decreasing the rate of splicing of a pre-mRNA to a mature mRNA that can be exported to the cytoplasm. Our evidence from both mapping of partially spliced G6PD RNA in the livers of mice and from the regulation of G6PD RNA reporters in rat hepatocytes indicates that splicing of the G6PD transcript is inhibited by polyunsaturated fatty acids. The decrease in transcript splicing results in a decrease in the production of mature G6PD mRNA in these cells. The cis-acting RNA element mapped to exon 12
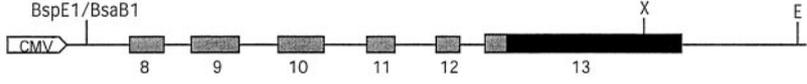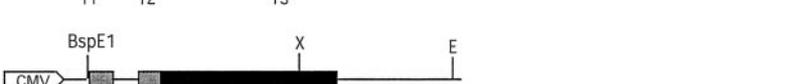
FIG. 8. **The cis-acting RNA element involved in the inhibition of G6PD expression by arachidonic acid localizes to exon 12.** Primary rat hepatocytes were transfected with the RNA reporter constructs shown in the figure, and the RNA was analyzed as described in the legend to Fig. 6. Each construct represents successive deletion of genomic DNA, resulting in the production of RNA representing smaller portions of the G6PD transcript except for pβ-gal ex12–13 and pcDNA 11–13, which represent the region of G6PD genomic DNA containing exon 12 ligated to a heterologous RNA or the G6PD cDNA, respectively. *Hatched boxes* represent G6PD exons, and the *solid box* represents the 3′-UTR. All constructs were driven by the CMV promoter. In these experiments, the hepatocytes were not co-transfected with RSV CAT. The percent inhibition by arachidonic acid (20:4) for the reporter construct was calculated by dividing the amount of reporter RNA in cells treated with glucose (25 mM) and insulin (0.1 μM) by the amount of reporter RNA in cells treated with glucose, insulin, and arachidonic acid (175 μM). All values were normalized to the amount of 18 S rRNA to correct for differences in total RNA amount. The values for endogenous rat G6PD expression were calculated in the same manner only using the protected fragment from the rat G6PD probe. The repetition for these experiments was pCMV in7–13 ($n = 3$), pCMV in9–13 ($n = 3$), pCMV ex10–13 ($n = 9$), pCMV ex11–13 ($n = 6$), pCMV ex12–13 ($n = 4$), pCMV in12–13 ($n = 3$) pβ-gal ex12–13 ($n = 4$), and pcDNA 11–13 ($n = 3$) individual transfection experiments.

of the primary transcript; the activity of this element required the presence of an intron adjacent to the exon. This does not appear to be a generalized effect of arachidonic acid on splicing because the pCMV1–5, 3′-UTR construct that contains G6PD introns 3, 4, and 5 but not exon 12 was not regulated by arachidonic acid. These results are consistent with the hypothesis than exon 12 contains an exon-splicing enhancer that is critical for the efficient splicing and ultimate formation of the mature mRNA. Arachidonic acid would then inhibit the activity of a splicing co-activator that binds this enhancer.

Regulated splicing often involves the activity of splicing enhancer elements in an exon or intron of an RNA. These elements are involved in the recognition of an exon in the RNA transcript (27). Most cases of regulated splicing involve alternative exon inclusion in the mRNA. Enhancer sequences in exons bind regulatory proteins that first define the boundaries of the exon and then recruit the spliceosome complex (28–30). These regulatory proteins are often members of the SR family of splicing activators (31). The regulated splicing of G6PD does not involve alternative exon inclusion; however, portions of this pathway may be common. For instance, splicing of G6PD pre-mRNA may require the presence of a splicing enhancer protein that binds to a sequence in exon 12 to facilitate spliceosome recruitment to the pre-mRNA.

Proteins involved in splicing have also been demonstrated to interact with components of the cleavage and polyadenylation machinery, thereby regulating this step in RNA processing (for review, see Ref. 32). The definition of a terminal exon involves not only recognition of the 3′-splice site at the start of the exon but also the polyadenylation signal at the 3′-end of the exon (33). Proteins binding at the polyadenylation site can enhance the efficiency of splicing of introns in that mRNA. To test if such an interaction occurred, which was specific to G6PD regulation, the SV40 polyadenylation signal was substituted for the G6PD signal and 137 nucleotides of the 3′-UTR. The presence of the G6PD polyadenylation signal alone was not sufficient to result in fatty acid inhibition of the reporter RNA from all constructs, in that expression of RNA reporters such as pCMVcDNA, 3′-UTR, and pCMV1–5, 3-UTR that contain the G6PD 3′-UTR were not inhibited by arachidonic acid. When the SV40 polyadenylation signal was included in the context of an RNA reporter containing intron 6 through exon 13, inhibition of G6PD mRNA by arachidonic acid was still observed, but the inhibition was attenuated compared with constructs with the intact G6PD 3′-end. Thus, the G6PD polyadenylation signal and/or intact 3′-end is not required for inhibition by arachidonic acid, but an interaction between this region and the splicing machinery cannot be ruled out. Nonethe-

less, inhibition of reporter RNA required introns 3′ of intron 6 in the G6PD transcript.

The presumed fate of the G6PD transcript in mice fed the high fat diet is degradation in the nucleus. Two pathways have been described for degradation of RNA in the nucleus. In the first pathway, RNA that is not completely processed does not leave the site of transcription in the nucleus (10–12). Specific proteins recruited to the RNA by the spliceosome complex most likely mark correct splicing (34–36). These proteins can then interact with the export machinery. In the absence of proteins to initiate export, the RNA can become a target for degradation. What remains to be determined in a multi-intron transcript is how the correct and complete splicing of all introns is detected, resulting in release of the transcript. In yeast, RNA that fails to acquire a poly(A) tail is retained at or near the transcription site and degraded by a complex of exonucleolytic enzymes called the nuclear exosome (10, 37). In mammalian cells, SRm160, a member of the SR family of proteins, promotes splicing, 3′-end cleavage, and nuclear release of transcripts (38). The efficiency of polyadenylation *per se* does not appear to be a primary determinant of G6PD mRNA fate (13). Alternatively, G6PD pre-mRNA retaining intron 11 may fail to acquire all the necessary proteins for release from the transcription site.

A second pathway of nuclear RNA turnover is used in the degradation of RNA containing premature termination codons. Certain mRNAs containing premature termination codons are degraded within the nucleus at the site of their transcription (39). This nuclear nonsense-mediated decay pathway appears to depend on the presence of either a Kozak consensus sequence in the mRNA or an internal ribosomal entry site. Scanning in the nucleus by some component(s) of the translational machinery may detect the premature termination codon and target the mRNA for degradation (40). Although G6PD mRNA does not contain a premature termination codon, the G6PD pre-mRNA retaining intron 11 contains an in-frame stop codon at the start of the intron and three stop codons within the now out-of-frame exon 12. To be consistent with this nuclear decay pathway, the mRNA should contain a Kozak consensus sequence. The G6PD pre-mRNA reporters containing intron 6 to exon 13 sequences or deletions thereof used in these studies do not contain a sequence with perfect identity to the Kozak consensus but do contain several in-frame ATGs, which may provide weak ribosomal entry, sites. Elucidating the further details of this pathway is the subject of current experiments in the laboratory.

Only a few examples have been reported of changes in the efficiency of splicing regulating gene expression. These include thymidylate synthase (41), tumor necrosis factor-α (42), and spot 14 (43, 44). Regulated expression of thymidylate synthase during the cell cycle requires both the thymidylate synthase promoter and a spliceable intron, which need not be from the thymidylate synthase RNA (41). In contrast, splicing of the tumor necrosis factor-α mRNA requires a cis-acting element in the 3′-UTR of the transcript (42). Thus, diverse mechanisms appear to be involved in this regulation. Splicing of the single intron on the spot 14 gene is enhanced by dietary carbohydrate, refeeding, and insulin (13, 43, 44), but the molecular details of this mechanism are not known. In addition to the increase in splicing efficiency, the transcriptional activity of the spot 14 gene is increased by these treatments (45). Regulating gene expression by both transcriptional and posttranscriptional mechanisms would result in a more rapid response to nutritional or hormonal changes. Regulation of the efficiency of splicing may be a ubiquitous mechanism for controlling lipogenic gene expression and may account for the discrepancies between changes in transcriptional activity and changes in mRNA abundance for several of these genes. Post-

transcriptional mechanisms for other lipogenic enzymes have simply not have been studied because of the large transcriptional changes of many of these genes (for review, see Ref. 1). G6PD provides an excellent model for these studies because polyunsaturated fat or other nutritional modifications do not regulate the G6PD promoter. Understanding the details of regulated splicing will provide new information about the myriad of mechanisms controlling gene expression.

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