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E2F-dependent mitogenic stimulation of the splicing of transcripts from an S phase-regulated gene

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ABSTRACT

There is one class of genes whose expression increases at the G1/S transition of the cell cycle. One of these genes codes for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), an enzyme that controls glycolysis. The cell-cycle regulation of the PFK-2 gene depends on a binding site for the transcription factor E2F located at the 5′ end of the first exon and involves not only a transcriptional, but also a post-transcriptional, mechanism. We have investigated this mechanism by studying in Rat-1 fibroblasts mature and immature mRNAs from the endogenous PFK-2 gene and from stably expressed transgenes containing PFK-2 gene regions. An increase in precursor mRNA half-life and processing took place at the G1/S transition. Transgenes with a mutated E2F binding site or with mutated splice sites lost the regulation by serum, indicating that both an intact E2F binding site and an efficient splicing reaction are necessary for proper mitogenic stimulation. In quiescent cells, the transgene lacking the E2F binding site was more efficiently spliced than the wild-type construct. These results indicate that, in the wild-type gene, precursor mRNA splicing is blocked in G0 and that this block requires the E2F binding site. The data provide evidence for a coupling between stimulation of promoter activity and increased mRNA splicing in the mitogenic regulation of S phase-regulated genes.

INTRODUCTION

When quiescent cells are stimulated to proliferate, the expression of several genes is induced at the G1/S transition of the cell cycle. These genes code for proteins involved in progression through the cell cycle, for enzymes of DNA synthesis and, as we have shown recently (1), for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), an enzyme involved in the regulation of glycolysis. The transcription factors of the E2F family play a central role in the cell-cycle transcriptional control of several of these genes, including the PFK-2 gene (1). It has been proposed that E2F complexed with the pocket proteins Rb, p107 or p130 represses transcription in the G0 and G1 phases and that this phosphorylation of the pocket protein in late G1 releases active E2F, thereby converting the E2F binding site of these genes into a positive element (reviewed in 2). Post-transcriptional mechanisms of control for cell-cycle regulated genes have also been observed at the level of transcriptional elongation (ribonucleotide reductase R2 subunit, 3), mRNA processing (thymidine kinase, 4; thymidylate synthase, 5), mRNA stability (PCNA, 6) and translation (thymidine kinase, 7).

We have characterized earlier in a PFK-2 gene (65 kb, 17 exons) the three promoters that control the synthesis of the fetal (F), muscle (M) and liver mRNA, respectively (8,9). The F mRNA is found in proliferating cells and its concentration decreases markedly when cells become quiescent or are induced to differentiate (1,9). F mRNA concentration increases in cultured fibroblasts stimulated by mitogens. We found (1) that the cell-cycle regulation of the F mRNA of PFK-2 depends on a binding site for the transcription factor E2F located at the 5′ end of the first exon. However, the increase in F mRNA concentration induced by serum in Rat-1 fibroblasts was higher than expected from the amplitude of the stimulation of the transfected F promoter linked to the luciferase reporter gene. This raised the possibility that serum controls F mRNA not only by increasing its transcription, but also by a post-transcriptional mechanism. We have now addressed this question by studying the effect of serum on the mature and immature mRNAs of the endogenous PFK-2 gene and on the mRNAs of transgenes stably expressed in Rat-1 fibroblasts. We show here that transition from the G0/G1 to the S phase of the cell cycle indeed involves an E2F-dependent increase in F mRNA processing and that this control occurs at the level of the splicing events.

MATERIALS AND METHODS

Cell culture and transfection

Rat-1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). For stimulation by serum, cells were first made quiescent in DMEM/0.5% FCS for 48 h, following which the medium was replaced by DMEM/10% FCS for the times indicated. For cell cycle analysis, cells were left in DMEM/0.5% FCS for 48 h (G0/G1 phase). They were then incubated in DMEM/10% FCS for 30 min or 6 h (G1 phase) or for 16 h in the presence of 300 μM mimosine (late G1 phase) or of 5 μg/ml aphidicolin (G1/S phase). After that, aphidicolin was washed and...
the cells were re-incubated in DMEM/10% FCS for 5 h (S phase) or for 20 h in the presence of 0.2 µg/ml nocodazole (M phase). We verified by bromodeoxyuridine incorporation that aphidicolin and nocodazole (1) as well as mimosine (data not shown) had the expected effect on the cell cycle. Pools of stable transfectants were obtained as described (1). Luciferase activity was assayed with a kit from Promega. In all the experiments, the absolute values of luciferase activity were at least 1000-fold the background (5 Lumac units) measured in extracts of non-transfected cells.

**Plasmid constructions and mutagenesis**

Plasmid p2KM2400luc has been described (10). To construct p2KM2400ΔEcoRI-BarII luc, the SalI (site in the polylinker)–EcoRI (made blunt end with the Klenow fragment of DNA polymerase I) fragment (–2400/+1495) and the BarII (made blunt end with the T4 DNA polymerase)–BglII (site in the polylinker) fragment (–1411/+41) were isolated from p2KM2400lac. The two fragments were inserted into pX2. Plasmid p2KM2400ΔStuI luc was constructed by excising the StuI fragment (–1289/−998) from p2KM2400lac and recircularizing the plasmid. To construct p2KM2400ΔStuI-AvaII luc, p2KM2400lac was digested with StuI (sites at –1289 and –998) and BglII (site in the polylinker). The remaining plasmid was ligated with the StuI fragment and the AvaII (made blunt end with the Klenow fragment of DNA polymerase)–BglII (site in the polylinker) fragment (–667/+41) isolated from p2KM952lac (10). To construct p2KM2400ΔSspI-MaeIII lac, the SalI (site in the polylinker)–SspI fragment (–2400/+639) was isolated from p2KM2400lac and the SalI–BglII luc in the polylinker fragment (–335/+41) was isolated from p2KM335lac (10). The two fragments were inserted into pX2. To construct p2KM2400Emut lac and p2KM2400AS-DSmut lac, site-directed mutagenesis was performed on p2KM2400lac by using the Chameleon double-stranded, site-directed, mutagenesis kit according to the manufacturer’s (Stratagene) instructions. The mutagenic primers (mutated bases underlined) were as follows: E2Emut (TCGACGCTTCGGGTATCAGGACAGGGGAGG), ASmut (GTCCCTTGAATTTACAAATACCAAGACCGGAGG), and DSamut (CCCTTCTTAATCTGTTAATAGCGG). All amplifications were performed with 1 µCi of [γ-32P]dCTP. After electrophoresis on a sequencing gel, the amplified products were quantified with a PhosphorImager (Molecular Dynamics) or an InstantImager (Hewlett-Packard).

**RESULTS**

**Relative concentration of F mRNA precursors during the cell cycle**

Our hypothesis was that the increase in F mRNA seen after stimulation by serum results not only from an increased rate of transcription, but also from an increased rate of processing of mRNA precursors that would otherwise be degraded faster. We had shown earlier (1) that the half-life of mature F mRNA (~90 min) is the same in serum-stimulated cells as in quiescent cells. We reasoned that an increased processing of the precursors into mature mRNA should result in a decreased ratio of precursor to mature mRNA. This of course holds true if the half-life of precursor mRNA is not shortened upon serum-stimulation, an assumption that was confirmed (see below). We therefore analyzed the mRNAs originating from the F promoter during the cell cycle. Rat-1 fibroblasts were made quiescent by a 48 h incubation in 0.5% serum or were synchronized at different stages of their cycle. Because of the very low abundance of F mRNA, we used reverse transcription-polymerase chain reaction (RT–PCR) to amplify, from nuclear or total RNA, fragments corresponding to the region that is specific for the F mRNA, namely between exons 1aF and 1M (Fig. 1A). By doing so, we
detected (Fig. 2A) not only the mature long (L) and short (S) F mRNAs, which result from the use of an internal splice site in exon 1aF (Fig. 1B), but also unspliced precursors containing the first two introns (Pr3) as well as precursors having retained intron aF (Pr1) or intron bF (Pr2) (Fig. 2A). Consistent with our earlier work (1), there was more mature mRNA in late G1 and thereafter than in G0/G1 (bands L and S in Fig. 2A). We calculated the ratio of precursors to mature products amplified from nuclear RNA during progression through the cycle. As shown in Figure 2B, this ratio decreased for all the precursors. We could not exclude that different amounts of cytoplasmic RNA could contaminate the different RNA preparations. To validate our finding, we therefore repeated the experiments with total RNA. The data in Figure 2C confirms that the ratio of precursors to mature F mRNA decreased upon stimulation by serum. These experiments support the hypothesis of a more efficient F mRNA processing in cycling cells than in quiescent cells.

We then quantified the relative concentration of the most abundant precursors (those containing intron aF; namely Pr1 and Pr3) in total RNA from Rat-1 fibroblasts synchronized at different stages of the cell cycle. We used a competitive RT–PCR technique with primers in exon 1aF and intron aF. When going from G0/G1 to G1/S, the level of mRNA precursors was increased, but to a lesser extent than mature mRNA (Fig. 3). As this transition is accompanied by an increased mRNA processing (see above), the increase in concentration of precursor mRNA measured here could reflect not only the increased rate of transcription, but also a decreased rate of degradation. We therefore compared the stability of these precursors in quiescent cells (G0/G1) and in cells stimulated by serum for 16 h (S phase). In each condition, cells were incubated in the presence of 0.5 µg/ml actinomycin D to block further RNA synthesis without affecting its processing (4). Total RNA was extracted at different times after addition of actinomycin D and the concentration of F mRNA precursor was measured by competitive RT–PCR. The half-life of F mRNA precursor was indeed longer in serum-stimulated cells than in quiescent cells, being ∼2.5 and 1 h, respectively (data not shown). These results show that, far from being less stable after serum-stimulation, precursor mRNA is actually stabilized. This, together with the increased rate of transcription, accounts for the increase in precursor mRNA concentration seen in cycling cells. As the production of mature mRNA can cope with this increase (Fig. 3), these data buttress our conclusion that processing is also increased under those conditions.

To investigate the mechanism of this post-transcriptional control, we studied the serum-regulation of constructs stably transfected in Rat-1 fibroblasts. These constructs included the PFK-2 gene region of interest placed upstream of the luciferase reporter gene. This region, which extended from the F promoter to the first 41 bp of exon 1M, contained exon 1aF; intron aF; exon 1bF and intron bF (Fig. 1).

**Serum-stimulation of luciferase activity of transgenes**

The transgene constructs (Fig. 1C) contained the wild-type region of the PFK-2 gene (construct a) and mutants thereof, either deleted within the introns (constructs c and e) or with a deletion of exonic sequences and of intron–exon boundaries (constructs b and d). Luciferase activity was measured in stably transfected cells made quiescent by a 48 h incubation in 0.5% serum or in cells subsequently incubated for 20 h in 10% serum (Fig. 1C).
Figure 2. Relative concentration of the F mRNA precursors during the cell cycle. Total or nuclear RNA was extracted from Rat-1 fibroblasts synchronized at different stages of the cell cycle as described in Materials and Methods. RT–PCR was performed on the RNA with primers corresponding to sequences in exons 1aF and 1M. (A) Autoradiogram of the RT–PCR products amplified from total RNA and map of the 5′ end of the gene showing the location of the primers used and the amplified products. L, long F mRNA; S, short F mRNA; Pr, precursor mRNAs; ns, non-specific. (B) Quantification, with a PhosphorImager, of the radioactivity in the precursor products (Pr) relative to that in the mature (L + S) products amplified from nuclear RNA. (C) Same as in (B) except that the products were amplified from total RNA.

An expected (1) serum-response was seen with the wild-type transgene (construct a). This response was also seen with the constructs deleted within the introns (constructs c and e). Our interpretation of the small amplitude of this response is given below. In contrast, the response was lost when exonic regions and their bordering splice sites were deleted, despite the presence of the E2F binding site in exon 1aF (constructs b and d). This suggested that exonic sequences or splicing elements, rather than sequences within the introns, are important for serum-regulation. We therefore mutated the splice acceptor (TAGG→AAAA) and donor (AGGTAAAC→AATTTAAC) sites bordering exon 1bF by site-directed mutagenesis (construct f). Stable transfectants of this construct lost the serum response, suggesting that the splicing reaction itself was important. We then studied a construct (g) in which the E2F binding site had been destroyed by a mutation shown earlier to prevent E2F binding (1). This construct showed no serum-response either. This was unexpected despite our previous demonstration (1) that stimulation of F promoter activity by serum depends on this E2F binding site. Indeed, destruction of the E2F site was expected to abolish only the part of the response due to the transcriptional effect of serum, but not that ascribed to increase in mRNA processing demonstrated above. These results indicated that proper growth-regulation of the F mRNA requires not only the promoter region with an intact E2F binding site, but also correct splicing events. They also suggested that the E2F binding site is required for the serum-stimulation of mRNA processing.

Serum-regulation of the splicing of the mRNA from transgenes

We therefore analyzed the effect of the different mutations on the splicing of the transgene mRNAs. Total RNA was extracted from the stable transfectants and submitted to RT–PCR. The products amplified between exon 1aF and the luciferase sequence were analyzed on gels (Fig. 4 A). Products corresponding to the mature long F mRNA and short F mRNA (L and S in Fig. 4 A) were present in cells transfected with the wild-type construct (a), with construct g where the E2F binding site has been destroyed (Fig. 4 A), and with the two constructs (c and e) deleted within the intronic regions (data not shown). Thus, the integrity of introns aF and bF and of the E2F site is not required for correct maturation of the mRNA. We observed with all these constructs two products (Lc and Sc in Fig. 4 A) ~270 bp longer than the products corresponding to the long F mRNA and to the short F mRNA. These additional products could correspond to the use of a cryptic acceptor splice site (CTTCA TGTTTCCAG/A) within intron bF at ~260 from exon 1M. A single product corresponding to the short F mRNA was present in cells transfected with construct b, as expected from
Figure 3. Cell-cycle regulation of the F mRNA precursors. Total RNA was extracted from Rat-1 fibroblasts synchronized at different stages of the cell cycle. Competitive RT–PCR for the mature (long and short F mRNAs) and precursor (Pr1 + Pr3) F mRNAs was performed as described at the bottom of the Figure. The relative mRNA concentration was expressed as the ratio of mature or precursor F mRNA over internal standard (St). A value of 1 was assigned to the ratio in cells in G0/G1.

deletion in this transgene of the splice site used for the long F mRNA (Fig. 4A).

In contrast, no mature product (bands L, Lc, S and Sc) was observed for constructs d (deletion of exon 1bF) and f (destruction of splice sites) despite the presence of the two donor splice sites in exon 1aF and of the acceptor splice site in exon 1M (Fig. 4A). Thus, splicing between these sites did not take place when the splice sites bordering exon 1bF were absent.

We then quantified the effect of serum on the abundance of the transgene products detected in these experiments. The mature products (L + S) derived from the wild-type construct a were induced 11-fold after serum-stimulation (Fig. 4A). This is similar to the increase in endogenous F mRNA concentration reported earlier (1). The mature product from construct b was induced by only ~3-fold. The apparent discrepancy between this strong serum effect on mRNA, seen with constructs a and b, and the smaller stimulation of luciferase activity is discussed below. The mature products from the properly spliced, but E2F site-defective, construct g were not induced by serum. This is consistent with the involvement of the E2F site not only in the transcriptional response to serum, but also in the postulated stimulation of mRNA splicing by serum. As to the mRNA precursors (Pr), they were induced ~2-fold (Fig. 4A). This was confirmed by using, in the competitive RT–PCR, primers (from intron bF and the luciferase sequence) that allowed a better quantification (Fig. 4B). All these data confirm that the increase in transcription seen upon stimulation by serum is coupled to a more efficient splicing in the S phase than in the G0/G1 phase. It is noteworthy that the mRNA from the constructs that could not undergo splicing was not (construct f), or was barely (construct d), induced by serum despite the presence of the E2F binding site (Fig. 4A and B). This fits with our earlier demonstration (1) that serum weakly stimulates the luciferase activity of a transfected construct in which the promoter region alone has been directly linked upstream of the reporter gene. This also indicates that the effect of serum on F promoter activity is weak as compared to the effect of serum on mRNA processing. The luciferase activity from the
wild-type transgene (construct a) was stimulated only 2-fold by serum (Fig. 1C) whereas the mature mRNA from this transgene was induced >10-fold (Fig. 4A). This could be explained by the presence and translation of mRNA precursor in the cytoplasm, which we actually detected by RT–PCR on cytoplasmic RNA. Moreover, the ratio of mRNA precursor to mature products measured in this way was comparable to that measured by RT–PCR on total RNA (not shown), indicating that unspliced precursor mRNA reaches the cytoplasm. The poorly induced luciferase translated from these precursors would consequently dilute the luciferase translated from the mature products. This could also explain the apparent lack of induction of luciferase activity (Fig. 1C) of construct b, whose mature product was induced to a lesser extent than that from the wild-type construct a (Fig. 4A).

**Serum-induced release from an inhibition of splicing in G0/G1**

The enhanced efficiency of the splicing reaction seen upon stimulation by serum could be due either to the stimulation of splicing at the G1/S transition or to the release from a block of this reaction in G0/G1. To address this question, we measured in quiescent cells and in cells stimulated by serum the ratio of unsplitted intact E2F binding site and an efficient splicing reaction compared to the intact E2F site. First, a comparison of products amplified by RT–PCR from RNA extracted from quiescent and serum-stimulated cells shows that processing of the F mRNA is also cell-cycle regulated (22). Secondly, the serum-regulation of splicing of the transgene mRNAs. RT–PCR was performed with primers in exons 1aF and in the luciferase gene (as described in Fig. 4A) on total RNA extracted from stable transfected cells (Fig. 1C) made quiescent in 0.5% serum for 48 h or subsequently stimulated with 10% serum for 17 h. An autoradiogram of the RT–PCR products is shown. L, long mRNA; S, short mRNA; Lc and Sc, long and short mRNA resulting from the use of a cryptic splice site; Pr, unspliced precursor mRNA. The ratio of the radioactivity in precursor mRNA to that in mature (L + S) mRNA is shown below each line of the autoradiogram.

**DISCUSSION**

Our previous studies showed that transcription from the F promoter is increased at the G1/S transition of the cell cycle. We found that this increase depends on an E2F binding site located just downstream of the two transcription start sites (1). We now show that processing of the F mRNA is also cell-cycle regulated and that this control involves the splicing step and requires an intact E2F site. First, a comparison of products amplified by RT–PCR from RNA extracted from quiescent and serum-stimulated cells shows that the relative concentration of the precursors diminishes as compared to that of the mature mRNA, despite an increased half-life of such precursors. Second, the serum-regulation of transfected transgenes corresponding to the F mRNA specific gene region linked to the luciferase reporter gene requires both the intact E2F binding site and an efficient splicing reaction, while the latter proceeds normally in the absence of the E2F site.

A serum-induced increase in the rate of mRNA processing has been described for thymidine kinase, whose transcription is also stimulated by serum in an E2F-dependent way (14,15). However, the mechanism of this post-transcriptional effect has not been established. The thymidylate synthase gene provides another example of regulation of mRNA processing by serum (5). For this gene, whose transcription is not stimulated by serum and for which a direct control by E2F has not been demonstrated, splicing signals, but not intronic sequences, are required for regulation as is the case for the F mRNA. The thymidylate synthase promoter region is necessary but not sufficient for this regulation since the latter is lost when the promoter is linked to a reporter gene, unless spliceable introns are included in the minigene.

Our work leads to a two-step model for the regulation of the expression of the F mRNA by serum. First, there is a transcriptional control exerted by the E2F factor. We showed earlier that in transfected cells in G0/G1 the activity of the F promoter is higher when the E2F binding site has been destroyed than when this site is intact (1). This suggested that in G0/G1 the promoter is repressed by E2F associated with a pocket protein. This negative control is similar to that described for the B-myb (16), E2F-1 (17–19), 53C2 (20), cyclin A (21) and thymidine kinase (22) genes. It has been proposed (20) that the binding of the E2F-containing complex interferes with the binding of components of the basal transcriptional machinery. The E2F binding sites in the genes mentioned above are indeed close to the transcription start sites, and this is the case for the F promoter. Alternatively, the E2F-containing complex would prevent transactivation by
upstream activators (23,24). In any case, stimulation of promoter activity at the G1/S transition would result from the lifting of this inhibitory mechanism following the dissociation of the pocket protein from E2F (2).

The second level of control exerted by serum in our model involves the splicing reaction and this requires the E2F binding site. Indeed, we showed that a transgene in which the E2F binding site has been destroyed is more efficiently spliced than the wild-type construct in G0/G1 and that its rate of splicing is no longer increased when the cells are serum-stimulated. Factor binding to the E2F site could therefore hinder the splicing reaction in G0.

A mechanism has been proposed that links mRNA processing to transcription (25,26). According to this view, a mRNA processing factor (for example, a SR protein) is recruited to the nascent transcript by the phosphorylated C-terminal domain of elongating RNA polymerase II. We speculate that this processing factor is first tethered by the transcriptional apparatus. Binding of the E2F-pocket protein complex in the vicinity of the transcription initiation site would interfere with this recruitment, either directly or (20) by preventing the binding of the transcriptional apparatus to the promoter. Alternatively, factors that bind to the E2F site could interfere directly with the splicing reaction by altering the behaviour of splicing factors. Such a post-transcriptional role has been attributed to the transcription factor Sp1-1/PU.1 (27) which can interact with the nuclear RNA binding protein, p54\textsuperscript{nucl}, suspected to be involved in RNA splicing. PU.1 impedes the binding of this protein to the RNA and alters the splicing process in vitro. In any case, this second level of control would provide an additional safety mechanism to prevent inappropriate expression of the PFK-2 gene in G0/G1. Thus, the mRNAs that escape the transcriptional block in G0/G1 are rapidly degraded, as we have shown here, because they are inefficiently spliced. Once the E2F-pocket protein repressor complex has dissociated at the G1/S transition, the transcription rate increases, the splicing reaction becomes more efficient and mature mRNA accumulates.

Our two-step model differs from the one put forward for the regulation of the thymidylate synthase gene. Ke et al. (5) proposed that a S phase-specific factor is attracted to the promoter and transferred to the nascent RNA which is then more efficiently spliced. Another difference between the F promoter and the thymidylate synthase promoter is that the latter is not regulated by the cell cycle (28). Different mechanisms of splicing control may thus coexist. Our model may account for the regulation of the expression of the thymidine kinase gene. The promoter activity of this gene is, like the F promoter, controlled negatively by E2F (22). At the G1/S transition, a change in thymidine kinase mRNA processing occurs. Very high molecular weight unprocessed hnRNAs decrease concomitantly with the appearance of mRNA intermediates of lower molecular weight, indicating a more efficient splicing (4). Thus, coupling of promoter activity and mRNA splicing could be a general mechanism of regulation of S phase genes.

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