MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5591–5595
0270-7306/90/105591-05$02.00/0

Vol. 10, No. 10

Ordered Splicing of Thymidine Kinase Pre-mRNA during the S Phase of the Cell Cycle

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Received 30 April 1990/Accepted 19 July 1990

Concomitant with the onset of S phase, a series of thymidine kinase (TK) splicing intermediates as well as mature TK mRNA accumulates in the nucleus of BALB/c 3T3 cells. Most of the TK splicing intermediates are retained by oligo(dT)-cellulose chromatography, and, therefore, 3′ end formation and polyadenylation probably precede the splicing of TK pre-mRNAs. We have further characterized the TK pre-mRNAs that are present in the nuclei of S-phase cells by using specific probes derived from each of the six TK intervening sequences. Based on the sizes of the pre-mRNAs and their patterns of hybridization with these intron probes, we propose a pathway for intron removal from nascent TK transcripts. Intron excision occurred by a preferred, but not necessarily obligatory, order which appears to have been conserved in mouse and Chinese hamster cells.

The cytosolic enzyme thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) is of particular interest for the study of cell cycle-dependent regulatory mechanisms, because the mRNA encoding this enzyme accumulates to high levels just prior to the onset of S phase (12, 15). The induction of TK mRNA at this point during the cell cycle involves transcriptional (4, 22, 24, 35), posttranscriptional (4, 10, 14, 21, 22, 26, 35) and translational (8, 9, 33) controls.

To obtain insight into the mechanism(s) involved in the posttranscriptional regulation of TK mRNA accumulation, we examined nuclear RNA from quiescent BALB/c 3T3 cells that had been restimulated to proliferate by the addition of fresh serum. Very little TK mRNA was detected in the nuclei of cells harvested during G_0 and G_1. At the onset of S phase, however, we observed a dramatic change in the processing of TK mRNA precursors (pre-mRNA) that was characterized by the appearance of a series of high-molecular-weight TK mRNA precursors in addition to mature TK mRNA. These high-molecular-weight TK mRNAs could be chased in the presence of the RNA synthesis inhibitor dactinomycin, suggesting a precursor-product relationship (11).

We proposed that the high-molecular-weight TK transcripts were splicing intermediates which retained various intervening sequences (IVSs). Most eucaryotic genes contain IVSs or introns that are excised from nuclear pre-mRNA by the RNA splicing machinery (1, 25, 27, 32). To gain insight into the nature of the putative high-molecular-weight TK pre-mRNAs present in G_1/S- and S-phase nuclei, as well as to determine whether there is a preferential order of IVS removal from the TK gene, we further characterized the TK pre-mRNA splicing intermediates present in S-phase cells by Northern (RNA) blot analysis.

Mammalian TK genes, which contain seven coding exons and six introns, have been well conserved during evolution (6, 20, 23, 31). We isolated unique sequence DNA probes from each of the six TK introns and used them to characterize the splicing intermediates in the nuclei. The human TK gene has the highest density of *Alu*-like repetitive elements within its intervening sequences of any gene that has been sequenced (6). The abundance of repetitive elements within the larger murine TK introns therefore presented a problem in obtaining unique sequence DNA probes. This fact, together with the large variation in sizes of the TK IVSs, resulted in probes of different specific activities. A scale diagram of the murine TK gene and the relative positions of each of the DNA probes used for the hybridization analyses is presented in Fig. 1.

Filters containing nuclear poly(A)$^+$ RNA from A31 cells were hybridized sequentially with probes derived from each of the TK introns or with the mouse TK cDNA probe (Fig. 2, lane Ex). The sizes of the pre-mRNAs detected were determined by comparing their migration distances with those of ^{35}S-labeled RNA markers that were synthesized in vitro, coelectrophoresed, and transferred along with the nuclear RNA samples (Fig. 2, lane M). The sizes of the major TK pre-mRNAs determined in this manner are much more accurate than the previous sizes that were estimated on the basis of the migration of the 28S and 18S rRNA bands (11).

The results shown in Fig. 2 are presented in order of the complexity of the hybridization pattern obtained with the designated intron probe. The probe for intron C hybridized strongly to the high-molecular-weight pre-mRNA, of 10.2 kilobases (kb) (Fig. 2, lane I-C). A single hybridization signal to this 10.2-kb band was also observed with the probe for intron F (Fig. 2, lane I-F). The weak signal obtained with this intron probe may be due to the fact that it was of relatively small size (120 base pairs) and, therefore, had a lower specific activity than most of the other intron probes. Alternatively, only a fraction of the 10.2-kb pre-mRNA may retain sequences from this small intron. The size of this large band is consistent with a pre-mRNA corresponding to the entire TK transcript inclusive of all exons and introns. Further evidence to indicate that the 10.2-kb transcript is, indeed, a complete unspliced TK pre-mRNA derives from the finding that all intron probes hybridized with this pre-

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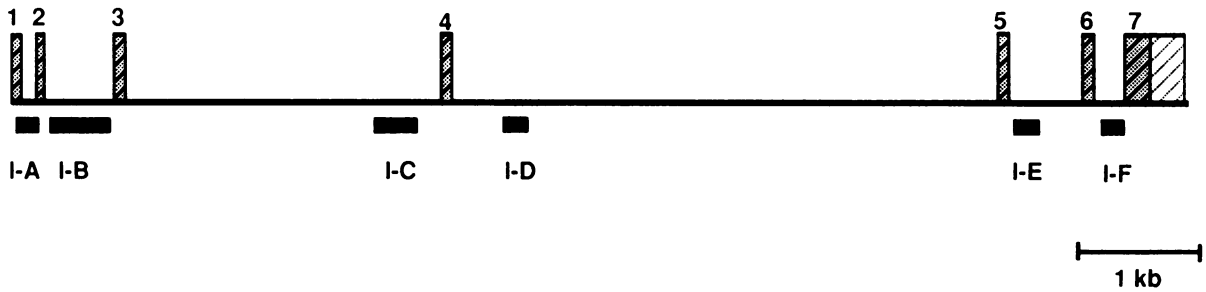

FIG. 1. Exon-intron organization of the murine TK gene and location of IVS probes. The numbers 1 to 7 above the hatched and dotted vertical projections represent the seven exon sequences of the TK gene. The open hatched vertical projection represents the 3′ untranslated region and the solid black line represents the intervening sequences. The filled rectangles designated I-A, I-B, I-C, I-D, I-E, and I-F represent the location and approximate size of the probes used to detect the presence of specific IVSs in nuclear TK pre-mRNAs.

mRNA. The streaks across the central portion of the filters designated I-F and I-A (Fig. 2, lanes I-F and I-A) lie outside the boundaries of the RNA lane and are due to nonspecific radioactive contamination.

The probe obtained from intron E hybridized to the two highest-molecular-weight TK pre-mRNAs, of 10.2 and 7.4 kb (Fig. 2, lane I-E). The probe derived from the largest IVS of the TK gene (Fig. 2, lane I-D) hybridized with pre-mRNAs of 10.2, 7.4, and 6.3 kb. The patterns of hybridization obtained with probes from the IVSs A and B were more complex. The probe for intron B hybridized to the two highest-molecular-weight pre-mRNAs and to a smaller pre-mRNA, of 2.6 kb (Fig. 2, lane I-B). The probe for intron A hybridized to the same mRNAs as observed for intron B; however, it also recognized a pre-mRNA of about 1.9 kb

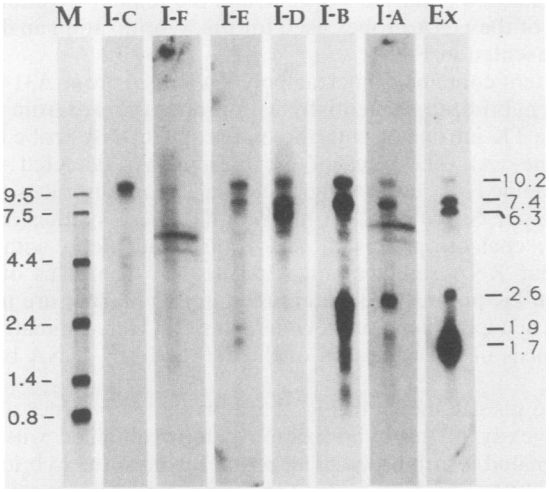

FIG. 2. Hybridization analyses of nuclear TK pre-mRNA with IVS probes. Nuclear RNA obtained from cells that had been made quiescent by serum deprivation and restimulated to proliferate for 18 h was fractionated by oligo(dT) cellulose chromatography. Poly(A)$^+$ RNA (10 μg) was size fractionated on a 0.75% formaldehyde-agarose gel, transferred to Nytran, and baked at 80°C for 2 h. Several lanes of poly(A)$^+$ RNA that had been coelectrophoresed and transferred were cut into individual strips and hybridized with probes derived from the respective IVSs: intron C (lane I-C), intron F (lane I-F), intron E (lane I-E), intron D (lane I-D), intron B (lane I-B), and intron A (lane I-A). The lane designated Ex is a representative filter that was stripped and rehybridized with a TK cDNA probe. The sizes (in kb) of the RNA markers (lane M) that were coelectrophoresed and transferred are indicated on the left. The sizes (in kb) of the TK pre-mRNAs are indicated on the right.

(Fig. 2, I-A). As expected, a cDNA probe containing only exon sequences hybridized with all of the TK pre-mRNAs as well as the mature mRNA (Fig. 2, lane Ex). The band at 1.9 kb was not seen as a discrete signal with the cDNA probe because of the abundance of mature TK mRNA at 1.7 kb.

A few minor bands which hybridized less strongly with the intron probes were also detected on the Northern blots (Fig. 2). These intermediates may be incorrectly processed RNA, or, alternatively, they may represent partially processed forms. The probes for introns E and B recognized several lower-molecular-weight RNA species that did not comigrate with pre-mRNAs detected by the TK cDNA probe. These bands may correspond to other unknown pre-mRNAs that share intron sequence homology with regions of the TK gene. Alternatively, they may be segments of TK introns, such as lariat intermediates, that have not been degraded. All of the intron probes used in these studies were shown not to hybridize with cytoplasmic RNA (data not presented) and, therefore, the low-molecular-weight species did not correspond to exon sequences.

TK mRNA is regulated in a cell cycle-dependent manner in Chinese hamster embryo fibroblast (CHEF) cells (J. M. Gudas, unpublished results). Furthermore, we have detected a similar ladder of high-molecular-weight TK pre-mRNAs in the nuclei of synchronized and exponentially growing CHEF cells. To compare the TK pre-mRNA bands present in CHEF cells with those found in mouse A31 cells, we isolated nuclear poly(A)$^+$ mRNA from exponentially growing CHEF cells that had been fed with fresh, serum-containing media 24 h prior to harvest. The CHEF nuclear RNA was hybridized with Chinese hamster cDNA sequences and probes derived from introns B and D of the Chinese hamster TK gene. We chose probes corresponding to these introns, since they had been shown to hybridize with a very distinct set of pre-mRNAs in mouse nuclei (Fig. 2).

A pattern of high-molecular-weight TK pre-mRNAs nearly identical to that seen in mouse A31 cells was observed in the nuclei of CHEF cells with a Chinese hamster cDNA probe (Fig. 3, lane Ex). When the same filter was rehybridized with a probe from the Chinese hamster intron D (Fig. 3, lane I-D), a strong signal to a pre-mRNA of around 6.9 kb and a weak signal to a pre-mRNA of approximately 8 kb were detected. Because IVS D is slightly larger in the Chinese hamster TK gene (20), these bands most likely represent the Chinese hamster counterparts of the mouse 6.3- and 7.4-kb nuclear precursors. The Chinese hamster intron B probe detected TK pre-mRNAs of 8.0 and 2.6 kb. The sizes of these precursors were almost identical to those detected by intron B in mouse nuclei. The TK pre-mRNA of

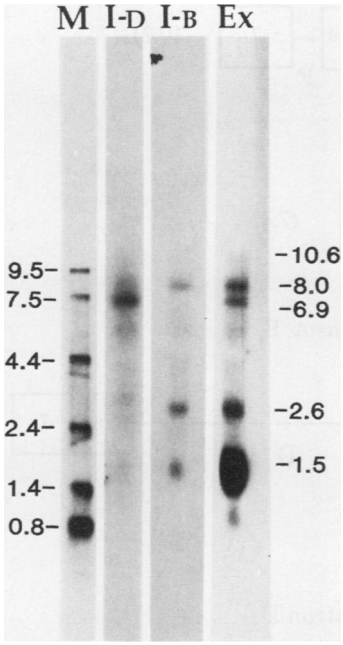

FIG. 3. Hybridization analyses of Chinese hamster TK pre-mRNAs. Nuclear poly(A)$^+$ RNA (10 μg) obtained from exponentially growing CHEF cells was size fractionated and transferred to Nytran as described in the legend to Fig. 2. The RNA was sequentially hybridized with probes derived from intron D (lane I-D) or intron B (lane I-B) or with a Chinese hamster TK cDNA probe (lane Ex). The sizes (in kb) of the RNA markers (lane M) that were coelectrophoresed and transferred are indicated on the left and the sizes (in kb) of the TK pre-mRNAs are indicated on the right.

10.6 kb, corresponding to a transcript from which no IVSs have been removed, was present at very low levels in the Chinese hamster nuclei. The intron probes probably did not detect this pre-mRNA because of their smaller size and lower specific activity.

The nearly identical patterns of TK splicing intermediates observed in the nuclei of both mouse and Chinese hamster cells argues that IVS removal from nascent TK transcripts was not cell type or species specific. Moreover, the striking similarities in TK pre-mRNA sizes and intron hybridization patterns suggests that the same preferred pathway for TK intron removal had been conserved between these two species. The murine and Chinese hamster TK genes each contain four large and two small introns. If subsequent IVS removal occurred randomly from the nascent transcript, we would theoretically have detected 24 precursors in the nucleus. This figure was calculated from the assumption that removal of only the four large introns would yield pre-mRNAs that differed sufficiently in size to be detected as distinct bands. The finding of only a few discrete TK pre-mRNAs with a cDNA probe, rather than the 24 predicted by random removal, thus implied that intron excision occurred via a preferred and orderly manner. The schematic depiction of the predicted splicing pathway presented in Fig. 4 reasonably fits our data and accounts for all the nuclear RNA species that were detected. Consistent with the size of the murine TK gene, as determined by DNA sequence analysis and restriction enzyme mapping, the largest detectable nuclear pre-mRNA that hybridized with all intron and exon probes was approximately 10.2 kb and thus probably represents a polyadenylated but unspliced TK transcript.

The intervening sequences C and F appeared to be removed either simultaneously or in an order that could not be predicted from our analyses to yield a pre-mRNA of approximately 7.4 kb (Fig. 4). The simultaneous removal of two intervening sequences has been reported to occur in vitro during the splicing of interleukin 3 pre-mRNA (17). Further processing of the 7.4-kb pre-mRNA likely occurred via two alternative pathways. Introns A, B, and E are spliced to produce the approximately 6.3-kb intermediate that retains the single large intron D (Fig. 4, right side). Subsequent removal of intron D, which is about 5 kb, would then yield an mRNA that is consistent with the size of the mature TK mRNA. Alternatively, introns D and E are spliced from the 7.4-kb precursor to yield the prominent 2.6-kb pre-mRNA that retains introns A and B, (Fig. 4, left side). Intron B appears to be excised at this point to yield the 1.9-kb pre-mRNA that retains only intron A. Subsequent removal of intron A would then yield the mature TK mRNA product (Fig. 4). The divergent pathway of intron removal is likely to be the preferred route, although it is probably not absolute (Fig. 4). Several of the faint bands detected by the probes for introns B, C, and E likely represent intermediates that arose from a different temporal order of intron splicing than the major pathway outlined above.

Our results are most similar to those of Tsai et al. (36), who reported that cleavage and polyadenylation precede the splicing of ovomucoid pre-mRNAs in chick oviduct nuclei. Furthermore, they determined that IVS excision for ovomucoid pre-mRNA occurs via a preferred pathway involving removal of introns E and F, removal of introns D and G, and, finally, removal of introns A, B, and C (36). The processing of intervening sequences from the primary transcript of the α_1-acid glycoprotein gene was also shown not to progress in a linear sequence from the 5′ end (34). In contrast, intron removal from phosphoenolpyruvate carboxykinase pre-mRNA was predicted to occur from the nascent transcript, beginning at the 5′ end and proceeding toward the 3′ end (13). By using a variety of techniques, other investigators have also demonstrated that the 5′ IVS of β-globin is preferentially excised before the 3′ IVS (5, 16, 17, 37).

The finding of different orders of intron removal for all pre-mRNAs examined rules out the possibility of a linear processive scanning mechanism (16, 19). However, these results are consistent with the first come, first served hypothesis for intron removal proposed by Abei and Weissmann (1). Present evidence indicates that each splice site of a multiple-intron-containing pre-mRNA is recognized independently by specific factors (2, 3). The subsequent interaction of these preestablished RNA-protein complexes then provides the appropriate juxtaposition of exons for their subsequent ligation to form mature mRNAs. Therefore, many different factors, including sequences at splice junctions (18, 19) and lengths and sequences of introns (7, 29) as well as sequences of adjacent exons (28, 30), probably contribute unique secondary structural features to pre-mRNAs which favor the interactions of specific spliceosome complexes over those of others. Many characteristics, including the overall intron-exon organization and the nucleotide sequences located at individual splice junctions, have been conserved in mammalian TK genes (6, 20). The observation that the same ordered pattern of intron removal has been preserved in mice, in Chinese hamsters, and probably in humans (J. M. Gudas, unpublished results) can most likely be attributed to secondary and tertiary structural features of nascent TK transcripts which favor the interactions of specific spliceosome complexes over those of others.

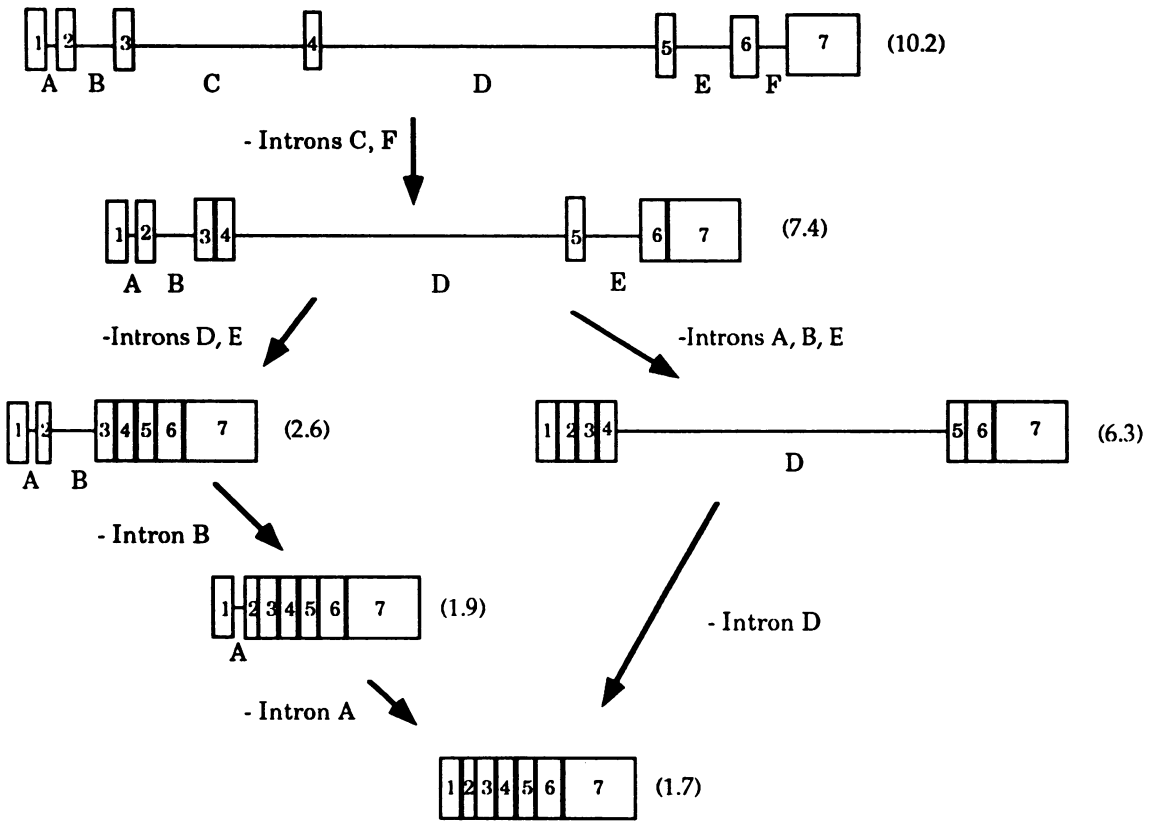

FIG. 4. Proposed order of intron removal from mouse TK pre-mRNA. Open boxes with numbers designated 1 to 7 represent the seven TK exon sequences. The six TK intron sequences, lettered A to F, are indicated by the black lines between the exons. The pathway for processing is shown by the arrows. The major intermediates detected by Northern blot analysis are indicated with their respective sizes (in kb) shown on the right.

This study was undertaken to provide a detailed characterization of the TK pre-mRNAs present in the nuclei of cells during S phase. Further investigation of the various processing steps involved in the formation of mature TK mRNA should further our knowledge of posttranscriptional regulatory events at the G_1/S boundary of the cell cycle. Moreover, these studies should increase our understanding of other, more global, regulatory mechanisms that operate to control the abundance of various mature mRNAs in the cytoplasm.

We thank Pin-Fang Lin for providing the mouse TK cosmid DNA and Mark Mercola for help and advice and for critical review of the manuscript. We are also grateful to Judith Fridovich-Keil and Deborah Zajchowski for their many helpful discussions and critical reading of the manuscript, to Jeffrey Goliger for helpful comments on the manuscript, and to Milton Datta and John Bryan for their expert technical assistance.

This research was supported by Public Health Service grant GM 24571 from the National Institutes of Health to A.B.P. and National Research Fellowship Award CA08317-03 to J.M.G.

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