The Cleavage/Polyadenylation Activity Triggered by a U-rich Motif Sequence Is Differently Required Depending on the Poly(A) Site Location at Either the First or Last 3′-Terminal Exon of the 2′-5′ Oligo(A) Synthetase Gene*

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Production of the two mRNAs encoding distinct forms of 2′-5′-oligoadenylate synthetase depends on processing that involves the recognition of alternative poly(A) sites and an internal 5′-splice site located within the first 3′-terminal exon. The resulting 1.6- and 1.8-kb mRNAs are expressed in fibroblast cell lines, whereas lymphoblastoid B cells, such as Daudi, produce only the 1.8-kb mRNA. In the present study, we have shown that the 3′-end processing at the last 3′-terminal exon occurs independently of the core poly(A) site sequence or the presence of regulatory elements. In contrast, in Daudi cells, the recognition of the poly(A) site at the first 3′-terminal exon is impaired because of an unfavorable sequence context. The 3′-end processing at this particular location requires a strong stabilization of the cleavage/polyadenylation factors, which can be achieved by the insertion of a 25-nucleotide long U-rich motif identified upstream of the last poly(A) site. Consequently, we speculate that in cells expressing the 1.6-kb mRNA, such as fibroblasts, direct or indirect participation of a specific mechanism or cell type-specific factors are required for an efficient polyadenylation at the first 3′-terminal exon.

Polyadenylation of nearly all eukaryotic pre-mRNAs is an obligatory step in the maturation of transcripts (reviewed in Ref. 1). The 3′-end processing occurs by cleavage of the precursor RNA, generating the upstream cleavage product that is elongated by a poly(A) tail (for review, see Refs. 2 and 3). The core elements of a polyadenylation signal correspond to a highly conserved hexanucleotide AAUAAA found 10–30 nucleotides upstream of the cleavage site and a less highly conserved GU-rich element located downstream of the cleavage site. Cleavage/polyadenylation is an intricate process requiring multiple cellular factors. At the first step of the polyadenylation reaction, the GU-rich sequence is recognized by one component of the cleavage stimulating factor (CstF),1 the protein CstF-64, which stabilizes the binding of the cleavage/polyadenylation specificity factor (CPSF) to the AAUAAA hexamer (2). Other factors that are required are the cleavage factors (CFIm, CFII m) (4, 5) and poly(A) polymerase. Finally, the Poly(A) binding protein II (PABII) specifies the correct length of the poly(A) tail and increases the efficiency of polyadenylation (6–8), enhancing mRNA stability and translation (9, 10).

Besides the core elements and secondary structures that influence cleavage/polyadenylation (11), auxiliary sequences located either upstream or downstream of poly(A) sites have been reported to influence 3′-end processing efficiency in a positive or negative manner. Upstream sequence enhancers (USEs) were primarily identified in viral poly(A) sites (12–16) and further in poly(A) sites of cellular genes, such as those encoding the complement factor C2 (17), lamin B2 (18, 19), and the secretory form of IgM (20). For these genes, the presence of USEs that are often U-rich increases polyadenylation efficiency. In contrast to U-rich sequences, the nature of downstream sequence elements (DSEs) is poorly defined and more complex. For example, it has been shown that a G-rich sequence downstream of the SV40 late poly(A) signal (21, 22) or a pseudo-exon sequence in calcitonin/calcitonin gene-related peptide (CGRP) transcripts (23, 24) simulates the processing at the upstream poly(A) site. For both USEs and DSEs, in vitro studies have indicated that they act as recognition sites for factors that stabilize the cleavage/polyadenylation complex (11, 14, 22). The presence of negative regulatory elements has also been described. In certain contexts, inhibition of polyadenylation was found to be associated with the presence of a splice donor site located either upstream or downstream of the poly(A) site (11, 18, 25, 26). For example, insertion of a bona fide 5′-splice site between the 3′-splice site and the poly(A) site was shown to abolish the coupling of the two reactions both in vivo and in vitro (27). In the case of the promoter-proximal human immunodeficiency virus-1 poly(A) site, it has been demonstrated that its usage is repressed by interaction of the U1 small nuclear ribonucleoprotein with a splice donor site almost 200 nucleotides downstream (28). Therefore, regulation of mRNA 3′-end formation may reflect the modulation of activities elicited by different regulatory sequences depending on

1 The abbreviations used are: CstF, cleavage stimulating factor; USE, upstream sequence enhancer; OASE, oligoadenylate synthetase enzyme; nt, nucleotide; CPSF, cleavage/polyadenylation specificity factor.

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growth condition of the cell, its response to specific stimuli, or its differentiated state. Ultimately, the net effect of these modulations will either mask or enhance suboptimal poly(A) sites leading to alternative polyadenylation (reviewed in Ref. 29). In addition, the efficiency at which the processing complex forms may also be influenced by the level of polyadenylation factors in the cell. Indeed, quantitative changes in CstF-64, possibly associated with the activities of not yet identified gene-specific regulators, have been reported to enhance recognition of an internal weak poly(A) site (30–32).

To gain more insights into the mechanisms leading to the cell type-specific selection of alternative poly(A) sites, we have investigated the processing of the pre-mRNA encoding the 2′-5′-oligoadenylyl synthetase enzyme (OASE) (33). In fibroblast cell lines, the OASE gene expresses 1.6- and 1.8-kb mRNAs (34, 35) that differ by distinct 3′-ends, termed pA1.6 and pA1.8, and the presence of a composite exon (Fig. 1A). However, in the B lymphoblastoid Daudi cells, only the 1.8-kb mRNA is detected. Based on transfection experiments using minigene constructs, we have compared the respective usage of the two poly(A) sites in different sequence contexts. We show that polyadenylation efficiency at the last 3′-terminal exon is independent of the poly(A) site sequence as well as of the surrounding regions. In contrast, although pA1.6 and pA1.8 carry a similar consensus core polyadenylation signal, substitution of one for the other at the first 3′-terminal exon showed functional differences in Daudi cells. Further experiments led to the identification within pA1.8 of a 25-nt long U-rich motif located immediately upstream of the AUAAAU signal that enhances cleavage/polyadenylation. Interestingly, insertion of this U-rich motif upstream of the pA1.6 poly(A) site is sufficient to induce an efficient cleavage/polyadenylation. These data add another example to the few mammalian poly(A) sites known to be regulated by such USE. However, in Daudi cells USE activity is not required to the same extent at the first and last 3′-terminal exons. Because the 1.6-kb mRNA is efficiently processed in fibroblast cells, these cells may contain some specific factors that allow 3′-end processing of pA1.6 in the absence of USE.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The primary Vwt plasmid was constructed by cloning the 4.4-kb SacI-HindIII genomic DNA fragment of the 2′-5′-OASE gene spanning from exon 4 to 1.1-kb sequence downstream of the terminal exon 8 (33) into the pGEMI vector. The minigene was placed under transcriptional control of the SV40 promoter obtained as a PvuII-EcoRI fragment from pSVK3 plasmid (Amer sham Biosciences). All other constructs used in this study were derived from the Vwt vector. In the Vd1.6, Vd1.8, and VppA plasmids, the pA1.6 and/or pA1.8 sequences (Fig. 2A) were duplicated or substituted for one another. Plasmids carrying the hybrid poly(A) sites at the first 3′-terminal exon (Figs. 3–5) were derived from Vd1.8 and Vwt and used as templates to generate oligo-mediated site-specific mutations with Thermo Pol Vent polymerase (New England Biolabs). The primer sequences and detailed procedures used for the design of the different constructs are available upon request. All the minigenes were sequenced using an Applied Biosystems sequencer.

**Cell Culture and Transfection**—Lymphoblastoid Daudi cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were maintained at 5 × 10^6 cells/ml at 37 °C and 5% CO_2. Cell transfection was carried out by electroporation of 2 × 10^7 cells in 0.3 ml of RPMI 1640 medium containing 20 μg of plasmid, using a Gene Pulser apparatus (Bio-Rad). Following the electroporation at 250 V, 960 μF, cells were resuspended in 20 ml of fresh complete medium and incubated at 37 °C and 5% CO_2 for 48 h. COS-7 cells were grown at 37 °C and 5% CO_2 in Dulbecco’s modified Eagle’s medium containing 8% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were grown to 60% confluency in 14-cm dishes and transfected with 5 μg of DNA using the FuGENE 6<sup>TM</sup> transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The medium was replaced after 16 h of incubation, and the cells were further incubated for 24 h at 37 °C and 5% CO_2.

**Poly(A)+RNA Purification and RT-PCR**—Transfected cells were washed twice with cold phosphate-buffered saline and harvested at 1500 rpm at 4 °C. Subsequently, the pelleted cells were used for poly(A)+RNA isolation by using the QuickPrep Micro RNA Purification kit (Amersham Biosciences). Purified poly(A)+RNAs were first subjected to DNase I treatment, and phenol/chloroform extracted and ethanol-precipitated. First strand cDNA was synthesized from 50 to 500 ng of purified mRNA primed with oligo(dT)_(12–18), using SuperScript II RNaseH-reverse transcriptase (Invitrogen). The OASE chimeric cDNAs were cloned by PCR using the following specific sets of primers: Fv1 (5′-GAGGCTTTTTGAGG-3′), R1.6, (5′-CACAATCGAGGTTGCT-3′); and R1.8, (5′-GTCAGCGTATGAGGAGG-3′). The Fv primer hybridizes immediately downstream of the last transcription start of the SV40 promoter. The R1.6 and R1.8 primers hybridize 45 and 25 nt upstream of the first AATAAA poly(A) signal of pA1.6 and pA1.8, respectively. Fv6, (5′-GTCGGAGGTCCTGCTAGGTG-3′), and R1.8, (5′-TGAATCPTGTTAATCAACATC-3′), were used to detect cDNAs corresponding to unprocessed chimeric RNAs. Fv6 and R1.8 hybridize within exon 6 and downstream of the ACCA cleavage site, respectively. PCR reactions were carried out as follows. The primers were annealed at 60 °C for 30 s, and the extension was performed at 72 °C for 45 s. The thermal cycling was achieved as indicated in the figure legends. PCR products were resolved on 1.5% agarose gels and visualized on a transilluminator after ethidium bromide staining.

**RESULTS**

**Cell Type-Specific Expression of the OASE Chimeric Gene**—Previous studies (33–35) have shown that maturation of the OASE RNA precursor involves alternative 3′-end processing that is regulated in a cell type-specific manner (Fig. 1A). To understand the mechanism underlying specific poly(A) site usage, we first set up an in vivo approach based on transient transfection of an OASE-shortened gene. We designed a construct, Vwt, in which the OASE genomic region spanning from exon 4 to 1.1 kb downstream of exon 6 was placed under transcriptional control of the SV40 promoter (Fig. 1B, see “Material and Methods”). Poly(A)+RNAs purified from different Vwt-transfected cell lines were reverse transcribed, and specific sets of primers were used in PCR reactions to amplify cDNAs corresponding to chimeric mRNAs (Fig. 1B). A plasmid expressing chloramphenicol acetyl transferase was used as transfection efficiency control (data not shown). As expected, monkey COS-7- and human FS11-transfected cell lines express chimeric RNAs polyadenylated at both 3′-terminal exons (pA1.6 and pA1.8 RNAs, respectively), whereas Daudi-transfected cells produce only the RNA processed at the last 3′-terminal exon (pA1.8, Fig. 1B). These results demonstrate that the chimeric pre-mRNA encoded by the Vwt minigene is submitted to the same cell type-specific maturation as the endogenous OASE precursor.

**Polyadenylation Efficiency of pA1.6 Is Differently Elicited at the First and Last 3′-Terminal Exons in Daudi Cells**—The 3′-ends of the pA1.6 and pA1.8 transcripts (Fig. 2A) share a ACCAUUUAAUG sequence that contains the CA cleavage site (33, 34). Interestingly, part of this motif is complementary to the consensus AUAAAU signal that is present in both 3′-regions. Despite the similarities of their core sequences, it was possible that the polyadenylation efficiencies of the two poly(A) sites depend on RNA cis-acting elements present in the surrounding regions. To investigate such a possibility, we first replaced the poly(A) site of pA1.8 with the corresponding region of pA1.6 (Fig. 2B, Vd1.6). We assessed the ability of the pA1.6 site to be amplified when present at the last 3′-terminal exon. Simultaneous RT-PCR amplification of the pA1.6 and pA1.8 RNAs was performed with the 3′ primers, R1.6 and R1.8, together with the common 5′ primer, Fv. As shown in Fig. 2B, Vd1.6-transfected Daudi cells produce accurately spliced RNAs ending at exon 6 as efficiently as pA1.8 RNAs in Vwt-transfected cells. As expected, in Vd1.6-transfected COS-7 cells, transcripts polyadenylated at 5% CO_2 for 48 h.
The meric transcripts purified from different transfected cell lines were transcriptional control of the SV40 promoter (boxes B-part of exon 5 (gray boxes)-terminal exon). The 3/H11032 denylated at either first or last 3/H11032-terminal exons are co-amplified at the last 3/H11032-terminal exon. To this end, we constructed two additional vectors (Vd1.6 and Vppa) that were used in transient transfection assays. The former carries a duplication of the poly(A) site of pA1.6 that was cloned in place of the corresponding region of pA1.6. In the second construction, the two sites were swapped with the pA1.8 site located upstream of the pA1.6 site. Because in Vd1.8 the sequence corresponding to the 3'/end of exon 5 (Fig. 4B, lane 3). However, no transcript was detectable when the remaining region was replaced by an irrelevant sequence (Fig. 3B, lane 11). These results imply that the 42-nt long sequence (a1.8) located immediately upstream of the AAUAAA signal activates pA1.8 processing when located at the first 3'-terminal exon in Daudi cells. Therefore, we conclude that the last 25 nt of a1.8 contain a sequence that promotes efficient usage of the pA1.8 poly(A) site when located at the first 3'-terminal exon. Because this element contains a high proportion of U residues (42%), we investigated the impact of their replacement by G residues (Fig. 4C). The mutation was performed in the context of the natural pA1.8 sequence, which allowed the use of the R1.8 primer. In cells transfected with the resulting VUG vector, transcripts processed at the first 3'-terminal position could not be amplified (Fig. 4C, lanes 3 and 4), which highlights the crucial role of the U residues in a1.8 for efficient poly(A) site usage. The position of the 25-nt long motif, immediately upstream of the AAUAAA signal, as well as its U-ricness fulfills the criteria defining USE, found in poly(A) signals of viruses and in a limited number of cellular genes (13, 15, 17–19).

**USE Activity Overcomes the Poor Usage of pA1.6 at the First 3'-Terminal Exon in Daudi Cells**—We further checked whether the USE of pA1.8 could trigger usage of pA1.6 at the first 3'-terminal exon in Daudi cells. Therefore, the a1.8 sequence, containing this element as well as the sequence of the R1.8 primer, was used to replace the corresponding region of pA1.6.
a1.6 (Fig. 5A). Consistent with the effect observed in the context of pA1.8, the USE strongly promoted usage of the pA1.6 chimeric site (Fig. 5B, lanes 3 and 4), whereas insertion of an irrelevant sequence had no effect (Fig. 5B, lane 5). Moreover, replacement of the core poly(A) site sequence, b1.6, or the downstream region, c1.6, with b1.8 and c1.8, respectively, did not generate transcripts processed at the first 3’-terminal exon (Fig. 5B, lanes 7 and 9) from the respective minigenes.

Overall, these results clearly demonstrate that the absence of the 1.6-kb mRNA in Daudi cells is because of the location of its last exon and the intrinsic weakness of its 3’-processing region. The unfavorable sequence context may require a strong stabilization of the cleavage/polyadenylation factors that can be promoted by the USE. Moreover, these experiments indicate that the USE functions independent of a particular core poly(A) site or specific downstream elements.

**DISCUSSION**

In this study we have investigated the sequence requirements underlying the use of two alternative poly(A) site sequences in the 2’-5’-oligo(A) synthetase gene. We have shown that the 3’-region of the first 3’-terminal exon (pA1.6), which is not recognized in the Daudi cell line, can be efficiently processed when inserted in place of the corresponding region (pA1.8) of the last terminal exon (Fig. 2B). This result demonstrates that the absence of the 1.6-kb mRNA in these cells does not result from intrinsic properties of its core polyadenylation site. The efficient cleavage/polyadenylation of pA1.6 at the

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**FIG. 2.** Differential 3’-end processing of pA1.6 in Daudi cells at the first and last 3’-terminal locations. A, sequences of pA1.6 and pA1.8 poly(A) site regions. The large black arrowheads indicate the cleavage sites. The AAUAAA polyadenylation signal and its complementary sequence are delineated by forward and reverse arrows, respectively. The common undecanucleotide sequence is in bold capital letters. Both poly(A) site regions were arbitrarily divided into three segments (a, b, and c). B and C, the schematic maps of the vectors bearing a duplication or a permutation of pA1.6 and pA1.8 are diagrammed. PCR reactions were performed at different cycles as indicated. PCR products corresponding to RNAs cleaved and polyadenylated either at the first or last 3’-terminal positions are pictured. The presence of unprocessed transcripts at the last 3’-terminal exon was analyzed by RT-PCR using forward (Fex6) and reverse (RG1.8) primers hybridizing within exon 6 and the region immediately downstream of the cleavage site, respectively.
3′-end of exon 6 likely is not because of the presence of positive elements in the surrounding sequences. The downstream region of this exon does not contain GU-rich motifs that are targets for CstF-64 or other accessory elements known to enhance cleavage/polyadenylation (reviewed in Refs. 1 and 2). Moreover, replacement of the complete sequence of exon 6 with an unrelated sequence, as well as deletions in the region downstream of the cleavage site, did not affect 3′-end processing of pA1.8. The Δnr corresponds to the replacement of the 25 nucleotides immediately upstream of the pA1.8 AUAAA polyadenylation signal by an irrelevant sequence. A, expression pattern of the minigenes carrying mutated pA1.8 sequences at the first 3′-terminal exons has to be increased of this transcript in COS-7 cells (data not shown). The Δa1.8 corresponds to a deletion of the first 17 nucleotides of the 5′-end of the pA1.8 region. This deletion removed the sequence corresponding to the R1.8 primer. Δnr corresponds to the replacement of the 25 nucleotides immediately upstream of the pA1.8 AUAAA polyadenylation signal by an irrelevant sequence. B, expression pattern of the minigenes carrying mutated pA1.8 sequences at the first 3′-terminal exons. Positions of the PCR products corresponding to mRNAs polyadenylated at the first (lanes 1, 3, and 5) and last 3′-terminal locations (lanes 2, 4, and 6) are indicated. C, effect of U mutation in the 3′-end processing of pA1.8. The V(U/G) construct presents, at the 3′-first terminal exon, a pA1.8 region in which U residues were substituted for G residues (asterisks mark these substitutions) within the 25-nucleotide sequence immediately upstream of the AUAAA polyadenylation signal.
elicitated, our results highlight the different requirements for a 3′-end processing enhancer element at these locations. The identification of such an element (Fig. 3B) was based on the finding that pA1.8 can be cleaved and polyadenylated when placed at the 3′-end of exon 5 (Fig. 2C). It corresponds to a 25-nucleotide-long U-rich sequence located immediately upstream of the polyadenylation signal of pA1.8 (Fig. 4). Insertion of this element in front of the pA1.6 poly(A) site promoted efficient polyadenylation at the 3′-end of exon 5 in Daudi cells (Fig. 5). This finding that pA1.8 can be cleaved and polyadenylated when placed at the 3′-end position abrogates its function (Fig. 4C), which confirms that U residues are crucial for USE activity (17, 19). Several lines of evidence suggest that USE may modulate polyadenylation through direct or indirect interactions with factors of the basal cleavage/polyadenylation machinery. For example, it has been shown that CPSF can bind to adjacent auxiliary elements such as AU-rich sequences (20). Similarly, the USE of the C2 complement gene (C2 USE) (17, 47) was shown to increase the affinity of CPSF-dependent binding of cleavage stimulating factor-64 to the C2 poly(A) site, leading to an efficient poly(A) synthesis. Moreover, C2 USE is also required for cleavage activity through its interaction with the polyypyrimidine tract-binding protein (PTB) (48, 49). Accordingly, it can be postulated that in Daudi cells interaction of the cleavage/polyadenylation complex with the internal poly(A) site requires the presence of a cis-acting sequence element such as USE to stabilize this interaction.

Therefore, in cells expressing 1.6-kb mRNA, such as fibroblast cells, 3′-end processing at the pA1.6 site must depend on a specific mechanism, possibly involving quantitative or qualitative cell type-specific differences in trans-acting factors. Supporting this hypothesis, our preliminary data have identified a 20-nucleotide-long sequence containing the internal 5′-splice site whose deletion strongly decreases pA1.6 usage without affecting cleavage/polyadenylation at the pA1.8 site when the latter is located at the internal position. Interestingly, this element and the pA1.6 region share specific binding activities that are differently distributed in cell lines expressing distinct ratios of OASE mRNAs. Therefore, it is possible that expression of the 1.6-kb mRNA in fibroblast cells requires interactions of specific splicing factors. Indeed, a 5′-splicing factor such as U1A bound to the proximity of a poly(A) site (14) was found to stabilize the interaction of the CPSF 160-kDa subunit with its AUA AAA-containing RNA substrate (50).

The observation (51) that in interferon-induced fibroblast cells derived from Alzheimer’s patients the processing of the 1.6 kb OASE mRNA is impaired confers to our model some interesting prospects. The characterization of the specific factors as well as the precise RNA targets involved in the 3′-end processing may help not only to gain more insights into post-transcriptional controls but also to reveal unexpected links with pathologies.

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