Length Increase of the Human α-Globin 3′-Untranslated Region Disrupts Stability of the Pre-mRNA but Not That of the Mature mRNA

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Polyadenylation increases the stability of mRNA molecules. By studying the effect of the length of 3′-untranslated region (UTR) on mRNA levels, we have found that α-globin pre-mRNA is stabilized by a mechanism that does not modulate the half-life of mature mRNA. The insertion of DNA fragments of various unrelated sequences into the 3′-UTR of the human α-globin gene strongly reduces mRNA abundance upon transfection into choriocarcinoma JEG-3 cells. We found an inverse relationship between mRNA levels and the length of the inserted fragments. In fact, mRNA levels as low as 1% were observed after inserting a 477-nucleotide (nt) fragment, whereas inserting a fragment of 86 nt at the same position had no effect on mRNA accumulation. DNA insertion induced no change in transcription rate or in half-life of mature mRNA. Semi-quantitative reverse transcription-polymerase chain reaction revealed that inserting a 477-nt fragment in the 3′-UTR resulted in decreased levels of nuclear pre-mRNA in proportion to that observed for mature mRNA. In contrast, the insertion of the 477-nt exogenous DNA in the last intron had no effect on mRNA levels despite the presence of intronic sequences in the pre-mRNA. This shows that the reduction of pre-mRNA level was not due to the insertion of putative ribonuclease cleavage sites or the insertion of a segment DNA that reduces the elongation efficiency. Taken together, our results strongly support the existence of a pre-mRNA stabilizing mechanism that can be disrupted by increasing the length of the 3′-UTR. The fact that the half-life of mature mRNA is not affected by DNA insertion is compatible with a pre-mRNA-specific stabilizing mechanism that acts specifically before polyadenylation.

Before reaching cytoplasm, most vertebrate primary transcripts are processed at their 5′ end by capping (1) and at their 3′ end by polyadenylation (2), and they are spliced to remove introns (3, 4). Pre-mRNA 3′-end processing is a two-step process that involves endonucleolytic cleavage and addition of 50–250 adenosine residues. 3′-End formation requires the presence of a polyadenylation signal (AA(U)UAAA) (5) and a G/urich sequence (6) localized upstream and downstream of the cleavage site on pre-mRNAs, respectively.

RNA 3′-end formation and splicing can occur independently in vitro (7–9). However, when introns were removed from genes, very low levels of mRNA accumulated upon transfection of culture cells (10–15) and in transgenic mice (16, 17). In fact, the presence of introns in pre-mRNAs increases levels of mature mRNA up to 50-fold by enhancing 3′-end formation (15, 18–22). It is now generally accepted that this effect depends on the splice acceptor site of the last intron (15, 18, 19, 23–28). Little or no effect on 3′-end formation was observed by deleting the donor site of the last intron indicating that the intron removal per se is not essential in 3′-end formation. This conclusion was reinforced by the observation that pre-mRNAs transcribed from two human β-globin genes defective in splicing were cleaved and polyadenylated as the normal β-globin pre-mRNA (29). A favored model to explain the role of the acceptor site is that spliceosome assembly at the 3′ splice site of the last intron may facilitate cleavage and polyadenylation processes (18, 19, 25, 26). We reasoned that if the enhancing effect of the intron on 3′-end formation involves an interaction between elements present in the two regions, then the length between the last intron and the polyadenylation site should influence the efficiency of 3′-end formation and, hence, the steady state levels of mRNA.

To study the interaction between a cis-acting element of the polyadenylation region and other element(s) located further upstream, we have increased the length of the terminal exon of the α-globin gene, more precisely, the length of its 3′-untranslated region (UTR), and analyzed mRNA steady state level. This was performed by inserting DNA fragments of various length and sequences. Co-transfection of the resulting clones with the parental plasmid into a choriocarcinoma cell line, JEG-3, that does not express the endogenous globin genes, was performed by inserting a 380-bp fragment bearing the small t intron and one ATTTA putative regulatory element, blunted, and re-circularized. Plasmid SV-wGL was prepared by inserting the 1119-bp HindIII-HindIII fragment of the SV40 large T-antigen gene between the HindIII sites of pSV-SPORT-1. Plasmids were transfected into JEG-3 cells by using the calcium phosphate-DNA precipitation method.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids—To prepare the pSV-SPORT-1Δ plasmid vector, pSV-SPORT-1 (Life Technologies, Inc.) was digested by Nhel and HpaI to remove a 380-bp fragment bearing the small t intron and one ATTTA putative regulatory element, blunted, and re-circularized. Plasmid SV-wGL was prepared by inserting the 1119-bp HindIII-HindIII fragment of the SV40 large T-antigen gene between the HindIII sites of pSV-SPORT-1. Plasmids were transfected into JEG-3 cells by using the calcium phosphate-DNA precipitation method.

1 The abbreviations used are: UTR, untranslated region; nt, nucleotide; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair; ORF, open reading frame; PIPES, 1,4-piperazinediethanesulfonic acid; ActD, actinomycin D; GM-CSF, granulocyte-macrophage colony-stimulating factor.
the entire intron 2 and exon 3 of
ments were prepared from the type 1 3
Not compassed the 3
183-bp probe encompassing the 3
Bal 477-S, -A (Fig. 1A), and the two smaller fragments were inserted into the Ball site of SV-gal to produce SV-gal/38-86-S, -A, SV-gal/3/286-S, and -A. Clone SV-gal/17β was constructed by inserting the EcoRI-StuI fragment of 295 bp, originating from the 5
1-globin (34) and the sequence remained in frame (Fig. 9A). The 3-UTR of this clone contains no α-globin sequences and starts at the first nucleotide of the NorI sites and sequences extends over the poly(A) signal present in the vector
templates, cells were harvested in cold phosphate-buffered saline and lysed in cold Nonidet P-40 lysis buffer (10 mM Tris (pH 8.0); 150 mM NaCl, 3
mM MgCl2, 0.1 mM EDTA) and centrifuged and used for nuclear RNA extraction (see below) or resuspended in storage buffer (50 mM Tris (pH 8.3), 40% glycerol, 5 mM MgCl2, 0.1 mM EDTA) and frozen in liquid nitrogen until nuclear runoff assays. Reactions were performed by adding 200 µl of nuclear suspension (1 × 107 nuclei) to 200 µl of reaction buffer (10 mM Tris (pH 8.0); 5 mM MgCl2, 0.3 mM KCl, 10 mM dithiothreitol, 1 mM each of ATP, CTP, GTP, and UTP [1000 µCi of [α-35S]GTP per reaction (Amersham)]). DNA probes for autoradiography were prepared by compiling PCR, and the 3′-UTR of these two clones contains the first 16 bases of the α-globin 3′-UTR. PCRs were performed with the Pwo enzyme (Roche Molecular Biochemicals), which has proofreading activity.

Other DNA clones were prepared as follows. The first step in the construction of clone SV-gal/17β was to linearize NorI site of the vector (5′-GGGGCCGGCGCGCCGACTAGTG-3′). In the resulting clone, the 1st NorI site was followed by a new glycine residue and the stop codon (Fig. 9A). The clone SV-gal dSmaI-NorI was produced by degradation of a DNA fragment prepared by total NorI digestion, followed by partial SmaI digestion of SV-gal to remove the 340-bp fragment containing exon 2 and part of introns 1 and 2. The 3′-HS3-1-PvuII-EcoRI fragment of 477 bp was inserted into the Ball site of these clones to produce clones SV-gal dINaI777, SV-gal dINaI777, and SV-gal dSmaI-NorI/477V.

The cloning scheme is shown. First, human blood was collected and centrifuged to remove serum and the white interface. The erythrocyte-enriched pellet was subjected to RNA extraction as described below, and first strand cDNA synthesis was performed using a Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia BioTech). Then, an aliquot of this reaction was subjected to PCR using a 5′ primer composed of a PstI restriction site, followed by sequences present in SV-gal, starting immediately downstream the PstI site of the Multiple Cloning Site (MCS) and ending at the 18th base of the α-globin gene (5′-GGGCTGCAATTCGCGTTTACCTAAGAGAAGGAC-CCAC-3′) and a 3′ primer composed of α-globin 3′-UTR sequences. Plasmid SV-gal was digested by PstI and then partially ligated with PstI. The resulting molecules were ligated to the PCR product. Molecular screening permitted isolation of the SV-gal/17β clone that contains exactly the same sequences as SV-gal but without introns.

The 3′-HS3-1-PvuII fragment of 477 nt was then inserted into the Ball restriction site to obtain SV-gal/17β.

Three different DNA preparations of each of the SV-gal, SV-gal/3/370-S, and SV-gal/3/477-S plasmids were used for transfections into JEG-3 cells without variation in their relative levels of expression.

Cell Culture and Transfections—COS-7 cells and the choriocarcoma cell line JEG-3 (ATCC HTB-36) were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, 25 mM HEPES, 50 units/ml penicillin, 50 µg/ml streptomycin, and 5% (v/v) JEG-3) or 10% (v/v) (COS-7) heat-inactivated fetal bovine serum (HyClone, Logan, UT). Cells were cultured as described previously (35-37). Cells were plated 18–24 h before transfection at a concentration of 2 × 106 cells per dish (10 cm) and transfected with the calcium phosphate procedure (38) using 1 µg of plasmid DNA per well.

RNA Extraction and Hybridization—Cellular RNA was extracted with TRI Reagent (39, 40) from pools of four dishes. RNAs were purified on CsCl gradients (41). The RNA samples were glyoxalized, electrophoresed, and gels transferred to Nytran Plus membranes (Schleicher & Schuell). Filters were hybridized at 42 °C and washed under high stringency conditions (33). Autoradiograms were quantified by densitometric scanning (Amersham Pharmacia Biotech RAS image analyzer system). The α-globin DNA probe was prepared using the 1119-bp HindII-Hinfl fragment of the α-globin gene. Clones with deletions in the ORF and those with a DNA insertion into the Ball site were also hybridized with a Ball-PstI 183-bp probe encompassing the 3′-UTR but lacking the open reading frame (ORF). Their levels of expression relative to SV-gal were the same as for the 1119-bp probe (data not shown). The γ-actin cDNA probe was composed of a 2-kilobase-pair cDNA fragment (42). All of the probes were labeled by random priming (43).

Nuclear Runoff Assay—After transfection with the selected plasmids, cells were harvested in cold phosphate-buffered saline and lysed in cold Nonidet P-40 lysis buffer (10 mM Tris (pH 7.5), 10 mM NaCl, 3
mM MgCl2, 0.3 mM EDTA) and digested by BstXI and NotI to remove the 3′-UTR, a part of exon 2, and the entire intron 2 and exon 3 of α-globin. The resulting fragment was ligated to a BstXI- and NotI-digested PCR product containing the α-globin sequence from BstXI to the stop codon inclusively, followed by a NotI site. The 5′ primer sequence containing the BstXI site of α-globin was 5′-CTGACACCCGGTGCTGGC-3′. The 3′ primer sequence containing the NotI site joined to α-globin sequences that begin at the stop codon and extend into the open reading frame (ORF) was 5′-GGGGCCGGCGCGTGTTCCAC-3′. The resulting DNA was named SV-gal/UTR-A (Fig. 4A). The 3′-UTR of this clone contains no α-globin sequences and starts at the first nucleotide of the NorI sites and sequences extends over the poly(A) signal present in the vector.

SV-gal was also digested by BstI and NotI, blunted, and ligated in the presence (SV-gal/3/370-S, SV-gal/3/477-S) or in the absence (SV-gal/3/370-S) of the 3′-HS3-477-bp fragment (Fig. 4A). The 3′-UTR of these two clones contains the first 16 bases of the α-globin 3′-UTR. PCRs were performed with the Pwo enzyme (Roche Molecular Biochemicals), which has proofreading activity.

Other DNA clones were prepared as follows. The first step in the construction of clone SV-gal/17β was to linearize NorI site of the vector (5′-ACAGATCGATGCTGGTG-3′), whereas the 3′ primer was composed of a BstXI site, followed by α-globin sequences encompassing the first 183-bp region of [α-35S]GTP (34). In the resulting clone, the second NorI site was joined to the 68th codon, and the sequence remained in frame (Fig. 9A). The clone SV-gal dINa2 was prepared by digestion of SV-gal by BstXI and NotI to remove the α-globin sequence downstream of the BstXI site and part of the downstream vector sequences. The resulting fragment was ligated to a PCR product digested by BstXI and NorI. The 5′ primer was composed of a BstXI site, followed by the α-globin sequence starting two nucleotides before the stop codon and extending into the 3′-UTR (5′-GGGCCAAACCGGTCTTCACGTCG-3′). The 3′ primer encompassed the NorI site of the vector (5′-GGGAGCGCCGGCGCCGATCAGTGTG-3′). In the resulting clone, the 1st NorI site was followed by a new glycine residue and the stop codon (Fig. 9A). The clone SV-gal dSmaI-NorI was produced by degradation of a DNA fragment prepared by total NorI digestion, followed by partial SmaI digestion of SV-gal to remove the 340-bp fragment containing exon 2 and part of introns 1 and 2. The 3′-HS3-1-PvuII-EcoRI fragment of 477 bp was inserted into the Ball site of these clones to produce clones SV-gal dINaI777, SV-gal dINaI777, and SV-gal dSmaI-NorI/477V.

The cloning scheme is shown. First, human blood was collected and centrifuged to remove serum and the white interface. The erythrocyte-enriched pellet was subjected to RNA extraction as described below, and first strand cDNA synthesis was performed using a Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech). Then, an aliquot of this reaction was subjected to PCR using a 5′ primer composed of a PstI restriction site, followed by sequences present in SV-gal, starting immediately downstream the PstI site of the

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control of the SV40 early promoter (Fig. 1A). Except when indicated, all plasmid constructs were derived from this parental clone, named SV-aGL.

To study specifically the effect of the length of the 3′-UTR on α-globin mRNA levels, transfections were performed in non-erythroid JEG-3 cells to eliminate possible effects of putative tissue-specific elements. The endogenous α-globin gene contains a CpG island extending both upstream and downstream from the transcription start site (44). As it has been suggested that CpG island DNA may increase transcription specifically upon integration in the genome, we performed transient transfections.

Two overlapping fragments originating from the type 1 3β-HSD cDNA clone were inserted in the unique BalI restriction site of the α-globin gene localized 14 bases downstream of the stop codon (Fig. 1A). The resulting clones, containing an insertion of DNA of 477 bp (SV-aGL/3β-477-S) or of 370 bp (SV-aGL/3β-370-S) in sense orientation, were each co-transfected with SV-aGL into JEG-3 cells. Their relative transient expression levels were analyzed by Northern blot. Although the insertion of the 370-bp 3β-HSD-1 fragment reduced the level of mRNA to 15% that of parental α-globin mRNA, the effect of the 477-bp 3β-HSD-1 fragment was more pronounced, leading to an mRNA level 1% that of α-globin mRNA (Fig. 1B). Similar results were observed in COS-7 cells (data not shown) indicating that the effect of DNA insertion is not specific to choriocarcinoma cells.

Effect of the Sequence and the Length of Exogenous DNA Inserts on α-globin mRNA Levels—To define the properties of putative inhibitory inserts, we first studied the same DNA fragments inserted in both orientations. Both orientations of the 477- and the 370-bp 3β-HSD-1 segments decreased mRNA levels to the same extent (Fig. 1C), showing that the effect of DNA insertion is orientation-independent. To determine whether the sequence of the DNA insert is important, a 295-bp cDNA segment originating from the ORF of the 17β-HSD-1 cDNA was cloned into the same BalI restriction site of SV-aGL. The sequence identity between the 3β-HSD-1 3′-UTR and 17β-HSD-1 cDNA fragments was less than 5%. Co-transfections with SV-aGL revealed that the 17β-HSD-1 fragment reduced mRNA levels to approximately that of the 370-bp 3β-HSD-1 insert (Fig. 1D). Thus, the inhibitory effects of DNA insertion are independent of the sequence of the DNA insert.

To determine whether the length of the DNA insert correlates with mRNA levels, we introduced two DNA fragments of 286 and 86 bp in both orientations into the BalI site of the α-globin gene. The four clones were each co-transfected with SV-aGL in JEG-3 cells. Clones containing the 477- or the 370-bp 3β-HSD-1 fragment in both orientations were included as controls. An example of a Northern blot with clones containing an insertion in the sense orientation is shown in Fig. 2A. The 86-bp 3β-HSD-1 fragment had no effect on mRNA levels in both sense and antisense orientations (Fig. 2B). This observation demonstrates that DNA insertion does not destroy any putative α-globin regulatory element encompassing the BalI site. Thus, the insertion of DNA per se does not explain the above observations. Moreover, a negative correlation was found between the relative expression of plasmids and the length of the DNA inserts (Fig. 2C). Therefore, the effects of DNA inserts on mRNA levels depend of the length of these inserted fragments.

The Effect of Exogenous DNA Insert Is Function of Its Localization in the α-Globin Sequences—We also examined whether exogenous DNA alters mRNA levels when introduced into the last intron. The SV-aGL/3β-INTRON clone was constructed in which the 477-bp 3β-HSD-1 fragment was inserted into the

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**Fig. 1.** Negative effect of insertion of DNA into the 3′-UTR on levels of α-globin mRNA. A, SV-aGL contains the 1119-bp HinII-HinII segment of human α-globin gene including the poly(A) signal. Exons correspond to boxes; introns and 3′-flanking sequences are shown by thick lines. Wavy lines and boxes represent vector sequences, and thick lines refer to 3′-HSD-1 cDNA fragments. Black boxes correspond to the ORF of α-globin, 5′- and 3′-UTR are represented by open boxes. All 3′-HSD-1 DNA sequences originate from 3′-UTR, except the small PstII fragment that is from the ORF. B, plasmids SV-aGL/3β-370-S (lane 1) and SV-aGL/3β-477-S (lane 2) were co-transfected with SV-aGL in JEG-3 cells. Cellular RNA was extracted 48 h later and Northern blot probed with α-globin DNA. Positions corresponding to each mRNA and their relative levels are shown. C, plasmids SV-aGL/3β-370-S and ΔA (lanes 1 and 2, respectively), and clones SV-aGL/3β-477-S and ΔA (lanes 3 and 4, respectively) were co-transfected with SV-aGL in JEG-3 cells. This experiment was performed as described in B. Arrows correspond to the orientation of DNA inserts into α-globin sequences. D, plasmids SV-aGL/3β-370-S (lane 1) and SV-aGL/3β-17β (lane 2) were co-transfected with SV-aGL in JEG-3 cells. This experiment was performed as described in B.

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**RESULTS**

An α-globin DNA, beginning in the 5′-UTR sequences and extending over the polyadenylation site, was placed under the control of the SV40 early promoter (Fig. 1A). Except when indicated, all plasmid constructs were derived from this parental clone, named SV-aGL.

To study specifically the effect of the length of the 3′-UTR on α-globin mRNA levels, transfections were performed in non-erythroid JEG-3 cells to eliminate possible effects of putative tissue-specific elements. The endogenous α-globin gene contains a CpG island extending both upstream and downstream from the transcription start site (44). As it has been suggested that CpG island DNA may increase transcription specifically upon integration in the genome, we performed transient transfections.

Two overlapping fragments originating from the type 1 3β-HSD cDNA clone were inserted in the unique BalI restriction site of the α-globin gene localized 14 bases downstream of the stop codon (Fig. 1A). The resulting clones, containing an insertion of DNA of 477 bp (SV-aGL/3β-477-S) or of 370 bp (SV-aGL/3β-370-S) in sense orientation, were each co-transfected with SV-aGL into JEG-3 cells. Their relative transient expression levels were analyzed by Northern blot. Although the insertion of the 370-bp 3β-HSD-1 fragment reduced the level of mRNA to 15% that of parental α-globin mRNA, the effect of the 477-bp 3β-HSD-1 fragment was more pronounced, leading to an mRNA level 1% that of α-globin mRNA (Fig. 1B). Similar results were observed in COS-7 cells (data not shown) indicating that the effect of DNA insertion is not specific to choriocarcinoma cells.

Effect of the Sequence and the Length of Exogenous DNA Inserts on α-globin mRNA Levels—To define the properties of putative inhibitory inserts, we first studied the same DNA fragments inserted in both orientations. Both orientations of the 477- and the 370-bp 3β-HSD-1 segments decreased mRNA levels to the same extent (Fig. 1C), showing that the effect of DNA insertion is orientation-independent. To determine whether the sequence of the DNA insert is important, a 295-bp cDNA segment originating from the ORF of the 17β-HSD-1 cDNA was cloned into the same BalI restriction site of SV-aGL. The sequence identity between the 3β-HSD-1 3′-UTR and 17β-HSD-1 cDNA fragments was less than 5%. Co-transfections with SV-aGL revealed that the 17β-HSD-1 fragment reduced mRNA levels to approximately that of the 370-bp 3β-HSD-1 insert (Fig. 1D). Thus, the inhibitory effects of DNA insertion are independent of the sequence of the DNA insert.

To determine whether the length of the DNA insert correlates with mRNA levels, we introduced two DNA fragments of 286 and 86 bp in both orientations into the BalI site of the α-globin gene. The four clones were each co-transfected with SV-aGL in JEG-3 cells. Clones containing the 477- or the 370-bp 3β-HSD-1 fragment in both orientations were included as controls. An example of a Northern blot with clones containing an insertion in the sense orientation is shown in Fig. 2A. The 86-bp 3β-HSD-1 fragment had no effect on mRNA levels in both sense and antisense orientations (Fig. 2B). This observation demonstrates that DNA insertion does not destroy any putative α-globin regulatory element encompassing the BalI site. Thus, the insertion of DNA per se does not explain the above observations. Moreover, a negative correlation was found between the relative expression of plasmids and the length of the DNA inserts (Fig. 2C). Therefore, the effects of DNA inserts on mRNA levels depend of the length of these inserted fragments.

The Effect of Exogenous DNA Insert Is Function of Its Localization in the α-Globin Sequences—We also examined whether exogenous DNA alters mRNA levels when introduced into the last intron. The SV-aGL/3β-INTRON clone was constructed in which the 477-bp 3β-HSD-1 fragment was inserted into the
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Fig. 2. Effect of the length of DNA inserts on levels of α-globin mRNA. Plasmids SV-αGL/3β-477-S, -A, SV-αGL/3β-370-S, -A, SV-αGL/3β-286-S, -A, and SV-αGL/3β-86-S, -A, were each co-transfected with SV-αGL in JEG-3 cells. Samples were processed as in Fig. 1. A, lanes 1–4 correspond to co-transfection of SV-αGL and the clones with the sense orientation of the 477-, 370-, 86-, and 286-bp inserts, respectively. B, relative mRNA levels of α-globin clones bearing DNA fragments of various lengths and orientations. The value of SV-αGL mRNA was arbitrarily fixed at 100%. Arrows indicate the orientation of inserts within the α-globin sequence. C, relative expression versus length of DNA inserts in nucleotides for clones bearing a 3β-HSD-1 DNA fragment into the restriction site BalI of the α-globin gene.

first half of the second intron of the α-globin gene (Fig. 3A). Messenger RNA from SV-αGL/3β/INTRON and SV-αGL had similar lengths, indicating that RNA splicing occurred correctly, albeit the presence of an insert into intron 2 (Fig. 3B). No difference in mRNA levels was observed between the clones after readjustment with γ-actin mRNA for the amount of RNA loaded per lane. These results demonstrate that the strong effect observed on α-globin mRNA levels by insertion of DNA fragments depends on its localization in the α-globin sequence. Moreover, because the presence of the 477-nt insert in the transfected plasmid does not necessarily correlate with low steady state mRNA levels, the decrease observed with SV-αGL/3β-477-S and -A compared with the parental plasmid is not an artifact caused by unequal transport of individual plasmid during transfection.

α-Globin-specific Regulatory Element Downstream from the Site of DNA Insertion—The fact that the length of DNA inserts determine the extent of the effect suggests that an interaction between two hypothetical cis-acting elements localized on each side of the BalI site is disrupted by DNA insertion. The next experiment was designed to determine whether an α-globin-specific regulatory element may be found downstream from the site of insertion. Sequences downstream from the stop codon of SV-αGL (Fig. 3A, SV-αGL/3β/INTRON) were deleted by joining the stop codon directly to the vector sequence, which contains its own polyadenylation signal originating from SV40. The replacement of the α-globin 3'-UTR by the 3'-UTR of the vector in both JEG-3 and COS-7 cells had no or only a weak effect on mRNA accumulation (Fig. 3B). Thus, in JEG-3 and COS-7 cells, no α-globin-specific regulatory element can be mapped within the 3'-UTR by substitution.

Does insertion of DNA also decrease the abundance of mRNA when all sequences positioned downstream from the site of insertion in the 3'-UTR are replaced by heterologous 3'-UTR sequences? To answer this question, plasmids SV-αGL-Δ3'UTR-B and SV-αGL-Δ3'UTR-B/3β-477-S were constructed (Fig. 4A). Results indicate that DNA insertion into the BalI site had similar effects on mRNA levels in both the α-globin and the vector 3'-UTR contexts (Fig. 4C). The only common features downstream from the BalI site between the two 3'-UTR might be the cis-acting elements involved in 3'-end formation. For this reason, if any cis-acting regulatory element positioned downstream from the site of insertion is disrupted by DNA insertion, this element might be included within sequences involved in 3'-end formation.

DNA Insertion and Promoter Activity—We verified whether insertion of DNA in the 3'-UTR also reduces expression of the α-globin gene under the control of its promoter. The 3β-HSD-1 477-bp fragment was inserted in both orientations at the BalI site of the plasmid pUCO, which contains the α-globin promoter.

Fig. 3. The effect of insert DNA depends on its localization in the α-globin sequences. A, the structure of SV-αGL DNA is shown on top. Symbols were described in Fig. 1. Clone SV-αGL/3β/INTRON contains the 477-bp PstII-EcoRI fragment from 3β-HSD-1 cDNA inserted at the indicated position in the antisense orientation (arrow). B, plasmids SV-αGL and SV-αGL/3β/INTRON were transfected separately in duplicate in JEG-3 cells. Samples were processed as described in Fig. 1 using the α-globin probe and the labeled γ-actin fragment.
and gene (30). Co-transfections in JEG-3 cells revealed that insertion of DNA in both orientations strongly reduced the expression of the α-globin gene under the control of its promoter (Fig. 5). We conclude that insertion of DNA had similar effects both in homologous and heterologous promoter contexts.

Nuclear runoff experiments were performed using clones SV-αGL-3'UTR-B and SV-αGL-3'UTR-B/3β-477-S. Although these two clones showed strong differences in mRNA accumulation (Fig. 4C), they produced signals of similar intensity in runoff assay (Fig. 6). This result indicates that insertion of DNA does not alter the rate of transcription.

**DNA Inserts Do Not Affect α-Globin Mature mRNA Half-life**—Is mature mRNA decay affected by insertion of DNA into the BalI site? We used two different approaches to examine this possibility. The first is based on the fact that two mRNAs with different half-lives, but transcribed from the same promoter, give a ratio close to 1.0 soon after transfection, but this ratio changes over time until the mRNAs eventually reach steady state levels. Each of the two clones, SV-αGL-370-S and -370-S, were co-transfected with SV-αGL, and their mRNA levels were analyzed at different times. We observed no variation in SV-αGL/370-S/SV-αGL or in SV-αGL/370-S/SV-αGL mRNA ratios over time (Fig. 7A). In fact, the ratios were independent of the establishment of mRNA steady state levels. Second, we did a time course experiment in the presence of actinomycin D (ActD) after co-transfection of SV-αGL/370-S and SV-αGL. SV-αGL/370 and SV-αGL mRNAs had very close t½ values, as evidenced by the stable mRNA ratio over time (Fig. 7B). These two observations indicate that the addition of an insert DNA into the BalI site of the α-globin gene did not alter mRNA decay.

**Levels of Nuclear Pre-mRNA**—We next planned an experiment to show whether the effect of DNA insertion occurs before RNA processing. RT-PCR was performed on nuclear RNA isolated from JEG-3 cells transfected with SV-αGL-3'UTR-B and/or SV-αGL-3'UTR-B/3β-477-S. To amplify specifically pre-mRNA, the 5' primer was located in the last intron, and the 3' primer was positioned 157 bases downstream from the polyadenylation signal (Fig. 8A). The presence of the 477-nt insert into the BalI site of the 3'UTR strongly reduced levels of nuclear pre-mRNA (Fig. 8B). It should be noted that when plasmid DNA was added in a negative RNA sample before the DNase I reaction that preceded the RT-PCR, no amplification product was observed (data not shown). Therefore, the addition of an insert DNA into the BalI site resulted in a proportional decrease of pre-mRNA and mRNA levels, indicating that the effect of DNA insertion occurs before RNA processing.

**α-Globin Regulatory Elements in the ORF and the Introns**—We next examined the possibility that the effect of DNA insertion may be related to an α-globin-specific regulatory element localized in the ORF or in an intron. Messenger RNA levels were only slightly lowered by deletion of DNA regions encompassing the intron 1 (SV-αGL Δin1) or intron 2 (SV-αGL Δin2) of the α-globin gene (Fig. 9, A and B). When the 477-nt fragment was inserted in the BalI site of these two clones, a strong decrease in mRNA accumulation was observed (Fig. 9C). The segment SmaI-NarI was then removed from SV-αGL. This deletion overlapped the deletion of clones SV-αGL Δin1 and SV-αGL Δin2. Again, the addition of the 477-nt insert into the BalI site strongly reduced levels of mRNA accumulation (Fig. 9C). Therefore, we failed to associate the effect of DNA insertion to the presence of any single α-globin specific sequence between the 10th nt of the ORF to the stop codon.

Because common features exist between introns as donor and acceptor sites, it is possible that the effect of the DNA insertion into the BalI site persists as long as one intron is present in the gene. To study this possibility, both introns were removed (clone SV-αGL-ΔDNA, Fig. 9A). The resulting clone had mRNA levels similar to that observed after the insertion of

![Image](https://example.com/image.png)
Disruption of \( \alpha \)-Globin Pre-mRNA Stability

Fig. 6. The presence of the 477-bp insert does not change the rate of transcription. Nuclear runoff assay was performed using nuclei isolated from JEG-3 cells transfected with SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B (dots 1 and 2 in A and B) or SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S (dots 3 and 4 in A and B). Dots 1–4 in A and dots 1 and 3 in B are SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B DNA, and dots 2 and 4 in B are \( \gamma \)-actin DNA. A and B are from two different series of experiments. A was of 1-h exposure at room temperature, and B was of 1-h exposure at \(-80^\circ\)C with a Lightning Plus intensifying screen. Substitution of SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B DNA for SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S DNA for dot blot also gave similar results (data not shown). C, transcription rate of plasmid SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B (no DNA insert) and SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S (+477) are presented after normalization to \( \gamma \)-actin using values obtained by PhosphorImager scanning of membranes autoradiographed in B.

Fig. 7. DNA insertion does not alter cytoplasmic mRNA half-life. A, plasmids SV-\( \alpha \)-Globin/3\( \beta \)-370-S (left) and SV-\( \alpha \)-Globin/3\( \beta \)-477-S (right) were co-transfected with SV-\( \alpha \)-Globin in JEG-3 cells. \( t_0 \) was fixed at 20 h after the addition of the calcium-phosphate precipitates. Northern blots were probed with \( \alpha \)-globin DNA. B, plasmids SV-\( \alpha \)-Globin/3\( \beta \)-370-S and SV-\( \alpha \)-Globin were co-transfected in JEG-3 cells. Cells were trypsinized, pooled, and re-plated after transfection to eliminate inter-variations. The next day, ActD (5 \( \mu \)g/ml) was added for 45 min, and RNA was extracted at different times. The \( t_0 \) corresponds to the end of the ActD treatment. Samples were processed as described in A.

Fig. 8. DNA insertion and pre-mRNA levels. JEG-3 cells were transfected with plasmids SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B and/or SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S, and nuclear RNA was extracted. A is a schematic representation of SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B pre-mRNA, and the position of the 477-nt exogenous sequences (thick line) present in SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S pre-mRNA is indicated. Black boxes correspond to ORF sequences; open box corresponds to 3'UTR, thin line to intron 2, and wavy line corresponds to RNA sequences present in pre-mRNA but removed by endonucleolytic cleavage during the polyadenylation process. Arrows show position of the oligonucleotides used in RT-PCR. RT reaction was performed using 5 \( \mu \)g of nuclear RNA and the specific 3' primer, and semi-quantitative PCR conditions were obtained using only 2% of RT reactions. B, analysis of RT-PCR end products by Southern blot using the 1119-bp Hinfl-Hinfi \( \alpha \)-globin probe. Because sequences localized downstream from the BalI site of these clones are not \( \alpha \)-globin sequences, the \( \alpha \)-globin probe hybridized specifically to a segment of the same length for the two RT-PCR products, which extend from the 5' primer to the BalI site. Fragments amplified from plasmids SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B and SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S were of 574 and 1051 bp, respectively. JEG-3 cells were transfected with SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B (lanes 1–3) and/or SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S (lanes 4 and 5). The relative intensity of specific signals are the same than those observed with ethidium bromide staining. Relative levels of SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B and SV-\( \alpha \)-Globin \( \Delta \)3'UTR-B/3\( \beta \)-477-S mRNAs were similar to these observed in Fig. 4 as determined by Northern blot analysis of the corresponding cytoplasmic RNA (data not shown).

mRNA accumulation (Fig. 9D). Therefore, the effect of DNA insertion is independent of the presence of intron sequences in the gene.

DISCUSSION

We have clearly demonstrated that increasing the length of the 3'-UTR by insertion of DNA segments had a strong negative effect on \( \alpha \)-globin mRNA level. In our model, neither the nucleotide sequence of the insert nor that of the 3'-UTR itself, excluding features common to all 3'-UTR, contribute to the observed effect. The only characteristic of the inserts that was related to the level of mRNA was their length.

We first postulated that increasing the length of the \( \alpha \)-globin 3'-UTR might disrupt an interaction between the acceptor site of the last intron and the polyadenylation site, thereby reduce polyadenylation efficiency and levels of mRNA. It is now clear that the strong decrease in mRNA levels observed by increasing the length of the 3'-UTR was not due to the disruption of such a mechanism because the effect of the length of the 3'-UTR was observed in the absence of intronic sequences as well (clone SV-\( \alpha \)-Globin-cDNA). In fact, insertion of DNA into the BalI site did not change the rate of transcription but decreased levels of pre-mRNA and mature mRNA proportionally. Therefore, our results strongly suggest that the \( \alpha \)-globin pre-mRNA was actively protected from degradation in the nucleus and

the 477-nt fragment into the BalI restriction site of SV-\( \alpha \)-Globin (Fig. 9D). However, the addition of the 477-nt insert into the BalI site of SV-\( \alpha \)-Globin-cDNA also strongly reduced levels of
that the insertion of DNA into the BalI site, but not within intron 2, had disrupted this mechanism of stabilization. The decrease in mRNA amount was not due to insertion of putative ribonuclease sites present on the exogenous DNA insert, as witnessed by the absence of effect of BalI restriction site used for DNA insertion. Substitution of the negative correlation between RNA abundance and the primary transcript (46). Therefore, pre-mRNA decay seems to be controlled at least for these genes.

We have demonstrated that α-globin pre-mRNA is stabilized and that this stabilization is responsible for an increase in α-globin mRNA of 100-fold or more. In addition, our results strongly suggest that this phenomenon is regulated by a mechanism that necessitates cooperation between two cis-acting elements localized each side of the BalI site. It is of considerable interest that this mechanism of RNA stabilization is specific to pre-mRNA, which would appear to represent a putative level of gene regulation.

**REFERENCES**

1. Shatkin, A. (1987) BioEssays 7, 275–277
2. Wahle, K., and Kühn, U. (1997) Proc. Natl. Acad. Sci. USA 94, 993–1005
3. Green, M. R. (1991) Annu. Rev. Cell Biol. 7, 559–599
4. Moore, M. J., Query, C. C., and Sharp, P. A. (1993) The RNA World (Gesteland, R. F., ed.) pp. 303–358, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Proudfoot, N. J., and Brownlee, G. G. (1976) Nature 263, 211–214
6. MacDonald, C. C., Wilusz, J., and Shenker, T. K. (1994) Mol. Cell. Biol. 14, 6647–6654
7. Kainer, A. R., Maniatis, T., Ruskin, B., and Green, M. R. (1984) Cell 36, 993–1005
8. Moore, C. L., and Sharp, P. A. (1984) Cell 36, 581–591
9. Moore, C. L., and Sharp, P. A. (1985) Cell 41, 845–855
10. Ash, J., Ke, Y., Korb, M., and Johnson, L. F. (1993) Mol. Cell. Biol. 13, 1565–1571
11. Gasser, C. S., Simonsen, C. C., Schilling, J. W., and Schimke, R. T. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6522–6526
12. Gruss, P., Lai, C.-J., Dhar, R., and Khoury, G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4317–4321
13. Hamer, D. H., and Leder, P. (1979) Cell 19, 1299–1302
14. Neuberger, M. S., and Williams, G. T. (1988) Nucleic Acids Res. 16, 6713–6724
15. Kamen, C. M. T.-F., and Göransson, C. M. (1990) Nucleic Acids Res. 18, 937–947
16. Brinster, R. L., Allen, J. M., Behringer, R. R., Gelineas, R. E., and Palmiter, D. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 836–840
17. Palmater, R. D., Sandgren, E. F., Avarback, M. R., Allen, D. D., and Brinster, R. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 478–482
18. Chiu, H. C., Dabrowski, C., and Alwine, J. C. (1991) J. Virol. 65, 6677–6685
19. Nesic, D., Cheng, J., and Maquat, L. E. (1993) Mol. Cell. Biol. 13, 3359–3369
20. Nesic, D., and Maquat, L. E. (1994) Genes Dev. 8, 363–375
21. Liu, X., and Mertz, J. E. (1993) Nucleic Acids Res. 21, 5256–5263
22. Pandley, N. B., Chodchoy, N., Tiu, T.-J., and Maruluff, W. F. (1990) Nucleic Acids Res. 18, 3161–3170
23. Miller, J. T., and Stoltz, C. M. (1992) J. Virol. 66, 4424–4431
24. Niwa, M., and Berget, S. M. (1995) Genes Dev. 5, 2086–2095
25. Niwa, M., MacDonald, C. C., and Berget, S. M. (1992) Nature 360, 277–280
26. Niwa, M., Rose, S. D., and Berget, S. M. (1990) Genes Dev. 4, 1552–1560
27. Villarreal, L. P., and White, R. T. (1983) Mol. Cell. Biol. 3, 1381–1388
28. Wasserman, R. M., and Steitz, J. A. (1993) Genes Dev. 7, 647–659
29. Antoniou, M., Geraghty, P., Hurst, J., and Grosveld, G. (1998) Nucleic Acids Res. 26, 721–729
30. Raymond, V., Atwater, J. A., and Verma, I. M. (1989) Oncogene Res. 5, 51–52
31. Lue-Tho, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J. L., Strickler, R. C., and Labrie, F. (1991) Mol. Endocrinol. 5, 1310–1312
32. Lue-Tho, V., Labrie, C., Zhao, H.-F., Couet, J., Lachance, Y., Simard, J., Leblanc, C., Gonde, J., Berube, D., Gagne, R., and Labrie, F. (1989) Mol. Endocrinol. 3, 1301–1308
33. Trembly, Y., Ringler, G. E., Morel, Y., Mohandas, T. K., Labrie, F., Straus, J. F., III, and Miller, W. L. (1989) J. Biol. Chem. 264, 20458–20462
34. Sambrook, J., Prish, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Beaudoin, C., Blomquist, C. H., and Tremblay, Y. (1995) Endocrinology 136, 355–364
36. Trembly, Y., and Beaudoin, C. (1993) Mol. Endocrinol. 7, 355–364
37. Trembly, Y., Fleury, A., Beaudoin, C., Vaille, M., and Belanger, A. (1994) DNA Cell Biol. 13, 1199–1212
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38. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
39. Beaudoin, C., Bonenfant, M., and Tremblay, Y. (1997) Mol. Cell. Endocrinol. 133, 63–71
40. Beaudoin, C., Blomquist, C. H., Bonenfant, M., and Tremblay, Y. (1997) J. Endocrinol. 154, 379–387
41. Tremblay, Y., Tretjakoff, I., Peterson, A., Antakly, T., Zhang, C. X., and Drouin, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8890–8894
42. Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H., and Kedes, L. (1983) Mol. Cell. Biol. 3, 787–795
43. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
44. Bird, A. P., Taggart, M. H., Nicholls, R. D., and Higgs, D. R. (1987) EMBO J. 6, 999–1004
45. Akahane, K., and Pluznik, D. H. (1992) Blood 79, 3188–3195
46. El Meskini, R., Boudouresque, F., and Ouafik, L. H. (1997) Endocrinology 138, 5256–5265