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Characterization of an unique RNA initiated immediately upstream from human α1 globin gene *in vivo* and *in vitro*: polymerase II-dependence, tissue specificity, and subcellular location

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ABSTRACT

We have identified an abundant transcript initiated upstream from the canonical cap site of human α1 globin gene in bone marrow cells and in COS-7 cells transfected with an α1 globin gene-containing plasmid. Similar to the major α1 globin transcript, this upstream RNA is present almost exclusively in the cytoplasm of the transfected COS-7 cells. It is also synthesized efficiently in vitro by RNA polymerase II in the nuclear extracts prepared from a Hela cell line and an erythroleukemia cell line, K562. RNAs isolated from these cell lines, however, do not contain this upstream transcript. The putative 5' end of the α1 globin upstream RNA is mapped by primer extension to base -45, which is located in between the CCAAT and TATA boxes. The synthesis of this RNA in vitro and in vivo, and the close proximity of its 5' end to the promoter of the α1 globin gene suggest a common mechanism regulating the transcriptional initiation of both the upstream and the major α1 globin RNAs.

INTRODUCTION

RNA transcripts that initiate upstream from the normal or canonical cap site have been identified in a number of eukaryotic gene systems (1-15). For example, both SV40 and polyoma viruses produce upstream RNA transcripts in vivo with 5' heterogeneity (1-4). More recent studies of the human β-like globin gene family have also revealed RNA species initiated upstream from the canonical cap sites of ε, γ, and β globin genes (7, 9-13).

For the human β-like globin genes, the majority of the upstream transcripts initiate no further than 220 bp upstream from the canonical cap site (for references, see Table 1). Approximately 5-10% of the β-globin RNAs in erythroid tissues are composed of these upstream transcripts (9-13). They are also synthesized in non-erythroid tissues (9, 14), in transfected cell lines (13, 14), and in an in vitro transcription system (11). A significant proportion of these upstream transcripts are capped, spliced, and polyadenylated (9-11).

Thus far, the mechanism responsible for the initiation of these upstream RNA transcripts without the apparent need for its eukaryotic CCAAT and TATA box promoter elements is unknown, as is the function or ability to produce normal polypeptides. To examine the generality of the upstream globin transcripts and their possible function in regulating the globin gene expression, we have extended the above study to the human adult α1 globin gene system. We report here the existence of a single upstream RNA transcript in bone marrow, in COS-7 cells transfected with α1 globin gene, and in nuclear transcription extracts. The 5' end of this transcript is mapped in between the "CCAAT" and "TATA" boxes, at a position 45 bp upstream from the canonical cap site of the α1 globin gene.

MATERIALS AND METHODS

Cell Growth and Transfection

The African green monkey kidney cell line CV-1 and the T antigen producing variant COS-7 were grown in Dulbeccos modified Eagles medium containing penicillin/streptomycin supplemented with 10% fetal calf serum (16). The erythroleukemic cell line K562 (GM5372A from Institute for Medical Research, NJ) was grown in RPMI 1640 medium containing penicillin/streptomycin and supplemented with 15% heat inactivated fetal calf serum (17). Induction of the K562 cells was accomplished by growth in the same media containing .05 mM hemin (Sigma) for 5 days or more (18). Both cell lines were maintained in a CO_2 incubator at 37°C.

Confluent monolayers of COS-7 and CV1 cell lines were transfected following the procedure of Mellon et al. (19), except that the cells were not glycerol shocked. Calcium phosphate-DNA precipitates containing 10 ug plasmid and 30 ug carrier were allowed to remain on the cells for 12-16 hours. Cells were then rinsed with media and fresh media added to the plate. Cells were harvested 48 hours after the addition of this fresh media.

RNA Isolation

RNA from K562 and transfected COS-7 cell lines was isolated as described by Maniatis et al. (20) but at a smaller scale. Briefly, cells were lysed in buffer containing .5% NP-40 and 10 mM Vanadyl-ribonucleoside complexes (Bethesda Research Lab). The cell lysate was transferred to a microfuge tube and underlayed with an equal volume of buffer containing 24% sucrose and 1% NP-40. Nuclei were pelleted

through the sucrose by centrifugation at 4°C in an Eppendorf microfuge for 5 minutes. The upper, cloudy, non-sucrose fraction was separated from the lower sucrose and nuclei pellet-containing fraction. Both fractions of RNA were purified by proteinase K digestion in 1% SDS, phenol/chloroform extraction, and ethanol precipitation.

Total RNA was isolated from bone marrow samples by lysis of the cells in guanidinium isothiocyanate (Fluka AG) followed by CsCl ultracentrifugation (20).

In Vitro Transcription of α1 Globin Gene in Nuclear Extract

Preparation of nuclear extracts from Hela cells and K562 cells, and in vitro transcription of the α1 globin gene were performed according to the conditions of Dignam et al. (21).

Standard transcription reaction (25 µl) contains 12 mM Hepes (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.75 mM DTT, 0.25 mM phenylmethyl sulfonyl fluoride, 10% glycerol, 0.25 mM each of ATP, CTP, GTP, and UTP, and 0.63 µg of plasmid DNA psvoα1p3d. Reaction mixtures were incubated at 30°C for 1 hr with or without the addition of α-aminitin at a concentration of 2 µg/ml.

To isolate RNA for primer extension analysis, the reactions were stopped by the addition of 5M urea, 0.5% SDS, and 10 mM EDTA, and samples were extracted once with phenol/chloroform. The organic phase was extracted with equal volume of 5 M urea, 0.35 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8), 10 mM EDTA, and 10 µg tRNA. Aqueous fractions were then pooled, re-extracted with phenol/chloroform and chloroform. Nucleic acids were precipitated in ethanol.

When the removal of DNA template was desired, the transcription reaction mixture was first digested with 80 µg/ml of DNAase I (Worthington, DPPF or DPRF grade) in the presence of 10 mM Vanadyl complexes at 30°C for 5 minutes. RNA was then purified by extraction and ethanol precipitation, and analyzed by primer extension.

RNAase Digestion

When the removal of RNA was desired, the transcription reaction mixture was digested with 180 µg/ml of RNAase A (Sigma) at 30°C for 5 min before extraction and ethanol precipitation.

Primer Extension Analysis of RNA

Primer extension of DNA probes along RNA templates using AMV (Avian Myeloblastosis Virus) reverse transcriptase (Life Sciences Inc.) was performed as described (22, 23). The 3' end-labelled DNA fragments were

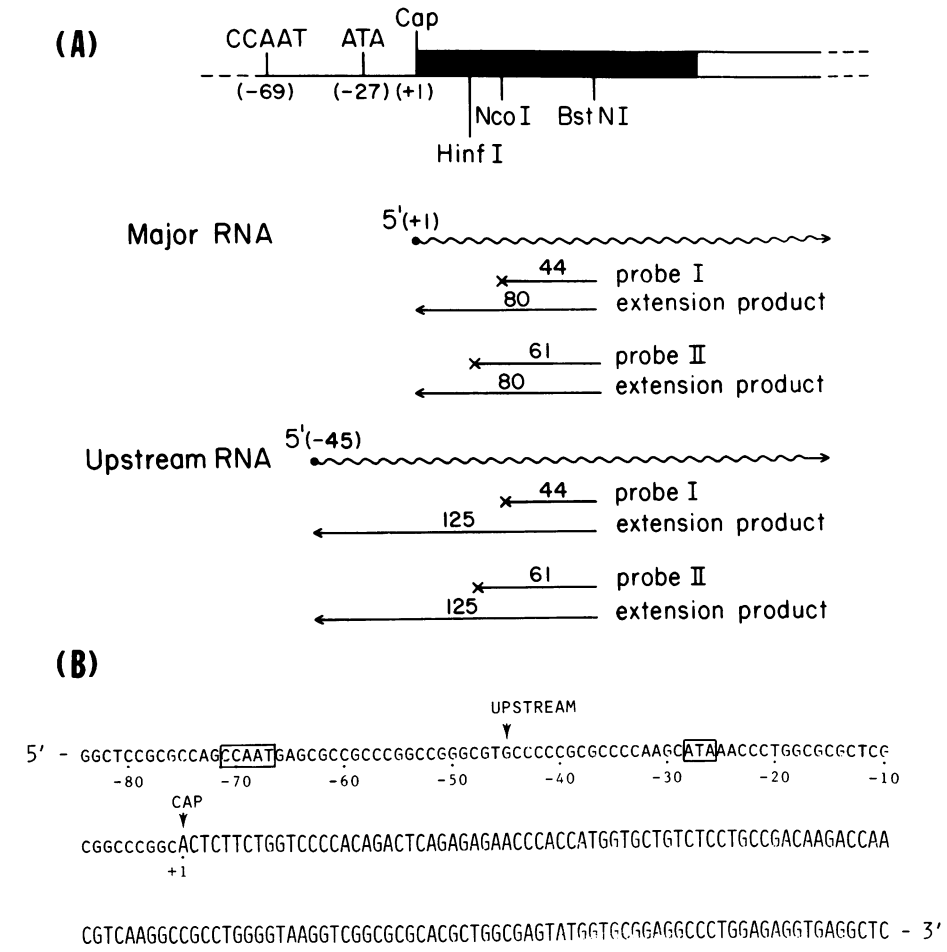

Fig. 1. (A) Strategy of primer extension. Shown on top is a restriction map of the first exon (the black box) of the human α1 globin gene and its 5'-flanking region. The positions of the CCAAT box, the TATA box, and the canonical cap site are indicated on the restriction map. The single stranded DNA primers used to hybridize with the RNA samples and their lengths (nt) are shown below the map. The signs "x" indicate the labeled 3'-ends of the primer: probe I, a 44 nt long, NcoI-Bst NI fragment; probe II, a 61 nt long, HinfI-Bst NI fragment. The lengths of the extension products along the upstream RNA and the major transcript as determined from Fig. 2 and 3 are also shown below each primer.

(B) Alignment of the putative initiation site of the upstream transcript with nucleotide sequence surrounding human α1 globin gene. The 5'-flanking sequence is represented by small letters. The promoter elements CCAAT and ATA are boxed. The 5' end (-45) of the upstream transcript (as determined in Fig. 2 and 3) and the canonical cap site (+1) are indicated by arrows.

prepared using the Klenow enzyme (Promega Biotec) and the appropriate α-^{32}P-dNTPs (3000 Ci/mmole, Amersham). Single stranded probes were purified by elution from 8% polyacrylamide-7 M urea gels.

RESULTS

An RNA Transcript Initiated Upstream From Human α1 Globin Gene Is Transported to the Cytoplasm of Transfected COS 7 cells

As a first step to detect upstream transcript from the human α1 globin gene, we have utilized the host-vector system devised by Mellon et al. (19). A plasmid, psvoα1p3d, which contains the human α1 globin gene within a 1.5 kb PstI fragment, is transfected into COS 7 cells. Replication of the plasmid in COS-7 cells allows the synthesis of large amounts of RNA for mapping analysis.

In the experiments described below, a 44 nt long, single-stranded NcoI-Bst NI probe labeled at the NcoI end was used as the primer (probe I in Fig. 1A). Hybridization of this probe to RNAs initiated at the canonical cap site is expected to give an 80 nt product after extension by the reverse transcriptase (Fig. 1A). Any transcript initiated upstream from the canonical cap site would result in a longer DNA product.

While nuclear RNA has very little signal, the cytoplasmic RNA isolated from the transfected COS-7 cells gives two autoradiographic signals after primer extension of the 44 nt long, NcoI-Bst NI probe (Fig. 2). The more intense doublet (80-81 nt) is the result of extension of the DNA primer along RNA transcripts that initiate at the canonical cap site. The less intense doublet (125-126 nt) corresponds to an RNA that initiates 45 nt upstream from the canonical cap site. Since the DNA probe was in excess, the relative intensities of the two doublets (5:1) represent the relative abundance of the two RNA species. Neither transcript was detected in CV-1 cells transfected with psvoα1p3d (data not shown), probably due to the low copy number of the plasmid in this cell line. The 5' end of this upstream transcript is indicated in Fig. 1B.

Mapping of α Globin RNA from Bone Marrow Cells

Following the above observation, we have examined total RNA from bone marrow cells using the same mapping technique. Primer extension analysis with the NcoI-Bst NI probe gave a similar band pattern as the cytoplasmic RNA of transfected COS-7 cells (compare Fig. 3A to Fig. 2).

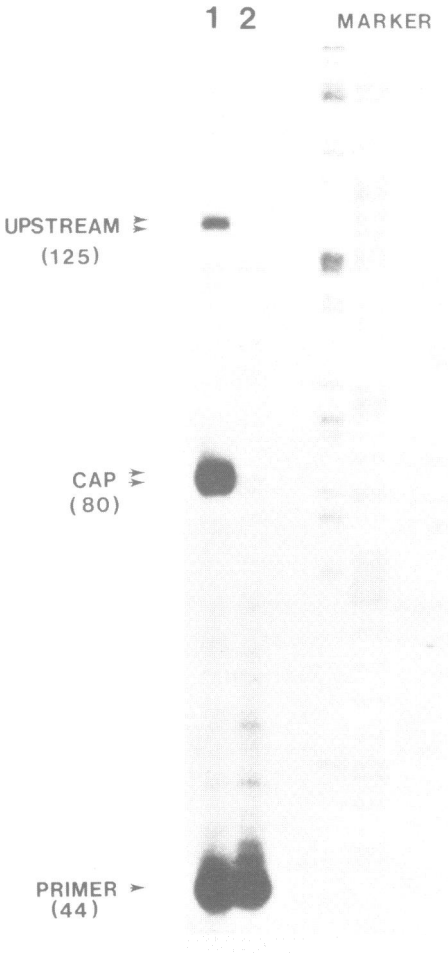

Fig. 2. Primer Extension Analysis of RNA Isolated from COS-7 Cells Transfected with Plasmid psvoαlp3d.

COS-7 cells were transfected with plasmid DNA psvoαlp3d as described in Materials and Methods. Cytoplasmic RNA (lane 1) and nuclear RNA (lane 2) were purified, and analyzed by primer extension technique after hybridization with the 44 nt long, single-stranded NcoI-Bst NI fragment (probe I in Fig. 1A). The extension products were analyzed by electrophoresis on 8% polyacrylamide gel-7M urea gel and autoradiography. The arrows indicate the positions of the primer, and extension products derived from the transcripts initiated from the canonical cap site, and the upstream site, respectively. As discussed previously (23), the lower band of each doublet represents species extending to the very 5' ends of the RNA templates. The 80-81 and 125-126 doubles were not seen after self-priming of the NcoI-Bst NI probe lane 1 (Fig. 3A), or in RNA samples isolated from COS cells transfected with carrier DNA only (data not shown). DNA sequencing markers were derived from pBR322.

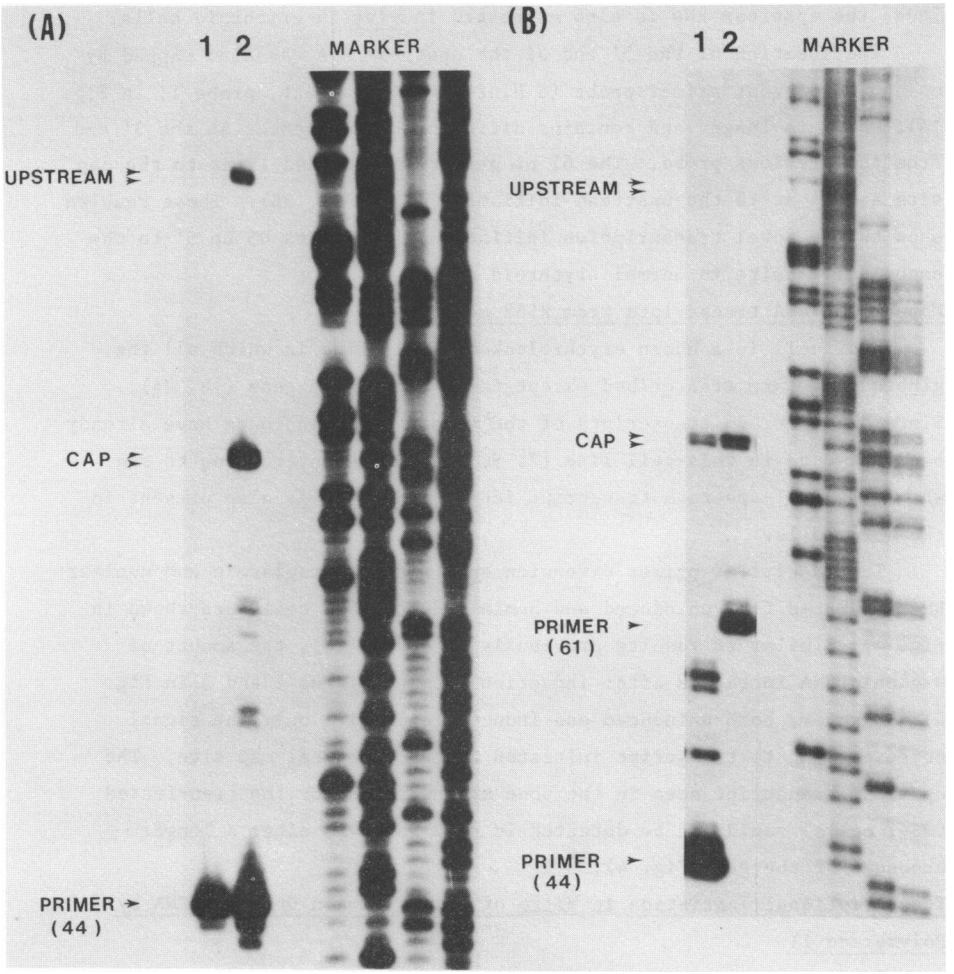

Fig. 3. Primer Extension Analysis of Total Bone Marrow RNA.
Total RNA isolated from bone marrow cells was analyzed by primer extension of two different probes.
(A) 1 µg of bone marrow RNA was analyzed by the 44 nt long, NcoI-Bst NI probe (probe I in Fig. 1A). Lane 1, self-priming of the probe; lane 2, extension product.
(B) 1 µg of bone marrow RNA was analyzed by primer extension technique using a 61 nt long, single-stranded HinfI-Bst NI fragment (probe II in Fig. 1A). Its extension products (lane 2) were compared to the extension products from the NcoI-Bst NI probe (lane 1) by electrophoresis on the same gel. DNA sequencing markers were derived from pBR322.

Thus, the upstream RNA is also expressed *in vivo* in erythroid cells.

The location of the 5' end of the upstream RNA was also mapped by using a different primer probe (a Hinf-Bst NI fragment, probe II in Fig. 1A), which is longer and contains different DNA sequences at the 3' end from the previous probe. The 61 nt primer is extended 19 nt to the cap site and 64 nt to the upstream initiation site (Fig. 3B). These results show that a novel transcription initiation site exists 45 bp 5' to the canonical capsite in normal erythroid cells.

Mapping of RNA transcripts from K562 cells

K562 cell is a human erythroleukemia cell line in which all the globin genes are transcribed except for the β-globin gene (18, 24). Since the upstream transcripts of the γ- and ε-globin genes have already been detected in this cell line (7, 9, 12), it is interesting to see whether the α1-upstream transcript identified above is also present in the K562 cells.

The results of primer extension analysis of cytoplasmic and nuclear RNAs isolated from uninduced and hemin-induced K562 cells are shown in Fig. 4. Similar to results previously observed (18), the amount of α-globin RNA increases after induction (compare lanes 1 and 3 in Fig. 4). However, both uninduced and induced cells give only the signal corresponding to transcript initiated at the canonical cap site. The upstream transcript seen in the bone marrow cells (or the transfected COS-7 cells) could not be detected in K562 RNA even after a longer exposure of the gel (Fig. 4).

Transcriptional Initiation *in Vitro* of the α1 Globin Upstream RNA by Polymerase II

To investigate whether the upstream α1 globin transcript can be generated *in vitro*, and whether RNA polymerase II or III is responsible for the initiation, we have mapped the 5' ends of α1 globin RNA transcribed from the plasmid psvoα1p3d in nuclear extracts prepared from Hela or K562 cells.

As shown in lane 1 of Fig. 5A, there are a number of bands generated after extension of the NcoI-Bst NI probe. This suggests that multiple initiation may occur *in vitro* upstream from the canonical cap site of the α1 globin gene. It is also possible that these multiple products arose from primer extension reactions on plasmid DNA fragments which contaminate the RNA preparation. Indeed, this was shown to be the case as shown in lane 2 of Fig. 5A. When DNAase I digestion was per-

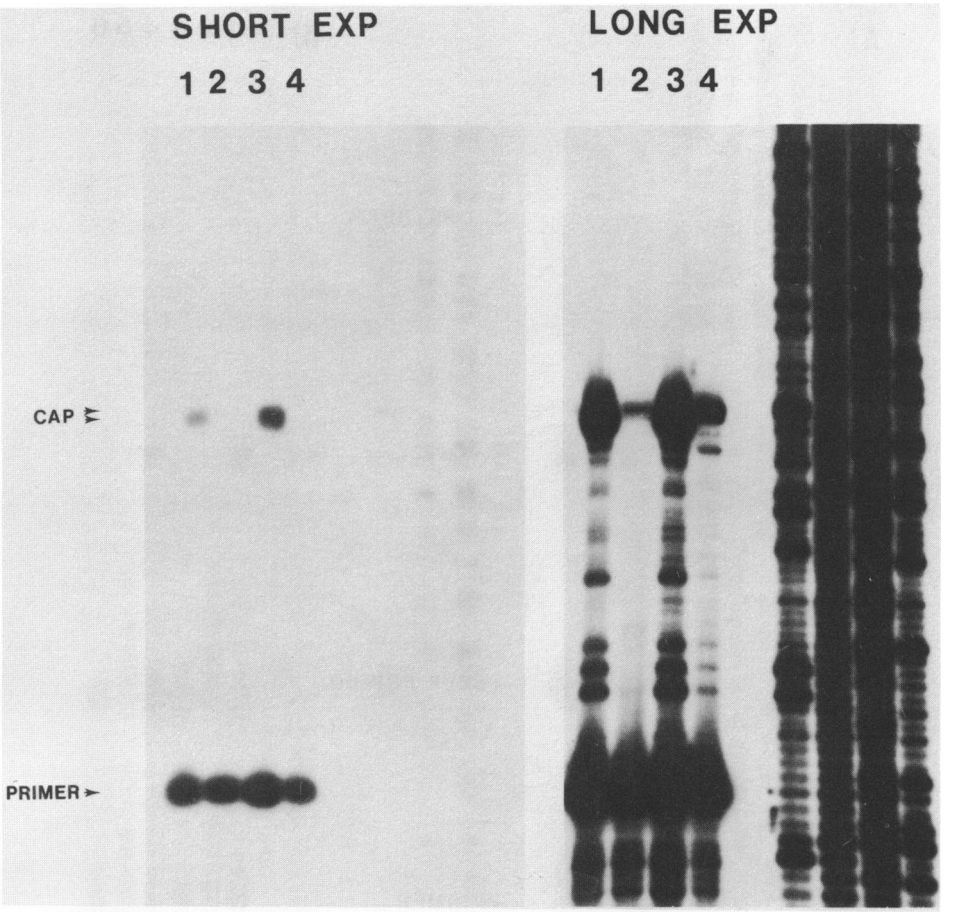

Fig. 4. Primer Extension Analysis of RNA Isolated from K562 Cell Line.
Cytoplasmic and nuclear RNAs from uninduced and hemin-induced K562 cells were analyzed by primer extension using the NcoI-Bst NI fragment as the probe. Lane 1, cytoplasmic RNA of uninduced K562 cells; lane 2, nuclear RNA of uninduced K562 cells; lane 3, cytoplasmic RNA of induced K562 cells; lane 4, nuclear RNA of induced K562 cells. The over-exposed autoradiograph on the right shows that no upstream α1 globin transcript is present in K562 cells. DNA sequencing markers were derived from pBR322.

formed before the RNA purification and primer extention, only two doublets were seen on the autoradiograph (compare lanes 1 and 2 of Fig. 5A). One of these doublets corresponds to RNA initiated at +1, and the other at -45. Their molar ratio is approximately the same as that of the bone marrow cells or transfected COS-7 cells.

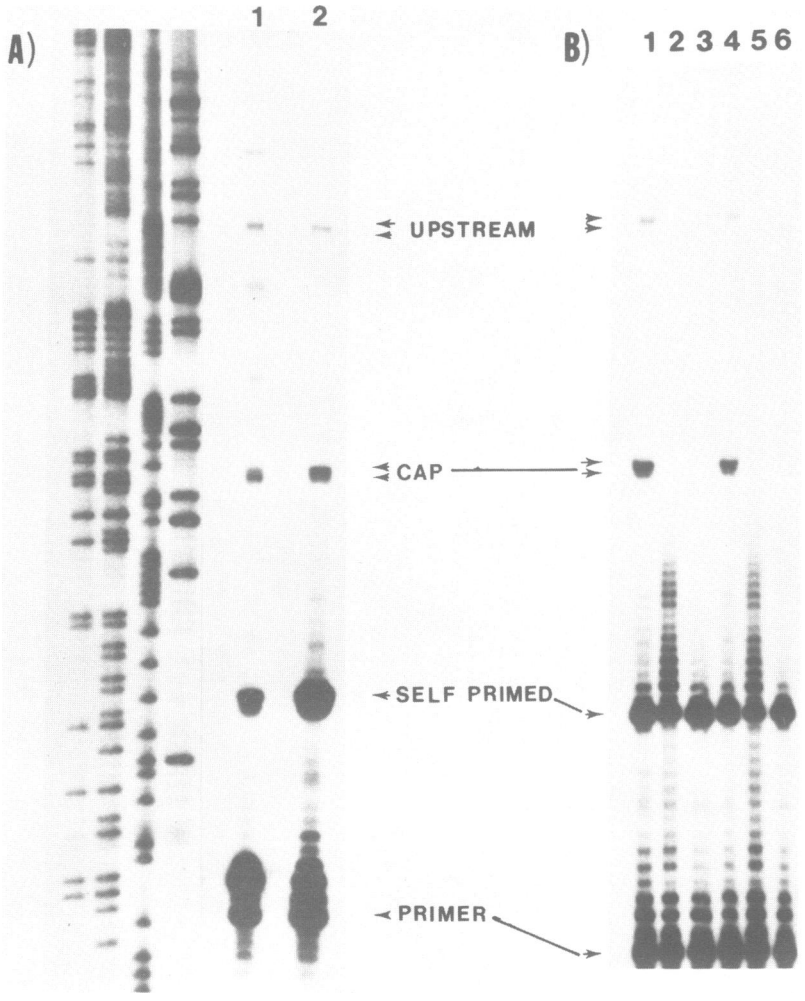

Fig. 5. Primer Extension Analysis of RNAs Transcribed from Plasmid psvoαlp3d in Nuclear Extract.

(A) Plasmid DNA psvoαlp3d was transcribed in Hela nuclear extract as described in Materials and Methods. After transcription, the nucleic acids were purified with (lane 2) or without (lane 1) prior DNAase I treatment, and analyzed by primer extension using the NcoI-Bst NI fragment as the probe.

(B) Plasmid DNA psvoαlp3d was transcribed in nuclear extract prepared from Hela (lanes 1-3) or K562 cells (lanes 4-6). After transcription, the reaction mixtures were digested with DNAase I, and the nucleic acid was purified for the analysis by primer extension of the NcoI-BstNI probe. Lanes 1 and 4, control samples; lanes 2 and 5, transcription mixtures were further digested by RNAase I before extraction and primer extension analysis; lanes 3 and 6, transcription was performed in the presence of 2 µg/ml α-amanitin. DNA sequencing markers were derived from pBR322.

Both the major and the upstream globin RNA appear to be synthesized *in vitro* by RNA polymerase II since the addition of 2 µg/ml of α-amanitin to the transcription mixture inhibits the initiation of both the -45 and +1 transcripts (lanes 3 and 6, Fig. 5B). As expected, RNAase digestion also eliminates both transcripts (lanes 2 and 5).

DISCUSSION

Our studies have demonstrated that the cells of bone marrow, an adult erythroid tissue, contains a minor RNA transcript whose 5' terminus is located 45 nt upstream from the canonical cap site of α1 globin gene (see map of Fig. 1B). The mapping technique used in this report is primer extension. Although the reverse transcriptase usually does not pause or stop at secondary structure(s) under our experimental conditions, we have performed S1 nuclease protection experiments in order to complement the primer extension approach. Negative results were obtained after using three different 5'-end labeled, single-stranded probes spanning the -45 position. A similar observation has been made by Treisman *et al*. (22). This is most likely due to the unusually high GC content (> 90%) of the region from -30 to -300 upstream from the α1 globin gene. We have also performed primer extention experiments using probes whose 3' ends are located at -112 or further upstream. All these probes gave negative results (data not shown). Thus, even if the detection of the -45 initiation site is the result of RNA secondary structure within the upstream transcript, the location of the 5' end of the transcript is unlikely to be beyond the base -112. Finally, we can not exclude the possibility of splicing although our nuclear extract lacks the RNA splicing activity under the *in vitro* transcription conditions used in Fig. 5.

This upstream transcript is also present in COS 7 cells transfected with an α1 globin gene-containing plasmid (psvoα1p3d). Interestingly, it is transported efficiently from nucleus to the cytoplasm of the transfected COS-7 cells (Fig. 2). We further showed that, when the plasmid psvoα1p3d was incubated in Hela or K562 nuclear extracts, accurate transcriptional initiation can occur at both the -45 position and the canonical cap site of α1 globin gene (Fig 5). This strongly suggests that the 5' end of the upstream transcript is an authentic initiation site *in vivo*. Some characteristics of the α1 globin upstream

Table 1. Summary of Upstream Transcripts from Human α, β, γ, and ε Globin Genes.

	Position of 5' end(s) relative to canonical cap site	Presence (+) or absence (-) in vivo and in vitro					
		Erythroid tissues	Cord blood/ peripheral blood	K562 cells	Transfected COS-7 cells[a]	In vitro transcription extract	Ref.
α	-45	+ (bone marrow)	ND	-	+ (cytoplasm)	+	this study
β	-176[b]	+ (bone marrow)	+	-	+ (nucleus)	+[c]	(10-13)
γ	-25 to -210	+ (fetal liver)	+	+	ND[d]	ND	(12)
ε	-200	+ (embryonic red cells)	-	+	+	ND	(7, 9)

[a]The subcellular location of the transcripts are indicated in the parentheses.

[b]A number of upstream transcripts have been mapped for the β-globin gene. However, discrepancy exists for many of these transcripts as mapped by different groups (10-13), and by the same group in two different studies (11, 13). The -176 species is the only RNA that has been consistently detected by all three groups. Some evidence suggested that RNA polymerase III may be responsible for the initiation of the -176 transcript (11).

[c]One study (11) showed that the -176 transcript was produced at very low level in vitro. Another in vitro study (10) showed no initiation from the -176 position.

[d]ND: not done.

RNA are compared to those of the human β-globin gene family as summarized in Table 1.

Tissue Specificity of Globin Upstream Transcripts

The detection of α1 globin upstream transcript in bone marrow cells (Fig. 3) suggests that the globin upstream transcripts are co-expressed in vivo with the canonical transcripts in erythroid tissues. As reported previously, the upstream RNAs of adult β- and fetal γ- globin genes are present in bone marrow and adult peripheral blood cells (10-13). The upstream γ-globin transcript can also be detected in total RNA isolated from the fetal liver, a fetal erythroid tissue (12). Similarly, the embryonic ε globin upstream transcripts were present only in embryonic erythroblast cells (7). Due to the heterogeneity of cell populations in the erythroid tissues, however, it is not known whether the upstream and canonical transcripts are synthesized separately in erythropoietic cells at different stages of differentiation.

Within the sensitivity of our assay (Fig. 4), we were not able to detect any α1 globin upstream transcript in K562 cells. This is not the case for the human embryonic and fetal β-like globin genes. The ε and γ globin genes produce both the upstream and the canonical globin transcripts in the erythroleukemia cells (7, 9, 12). (Note that the adult β-globin gene is not transcribed at all in K562 cells (10, 12, 24).) The different pattern of upstream initiation of different globin genes in this cell line may reflect a basic difference in the transcriptional mechanism of adult vs. embryonic/fetal globin genes, which in turn may result from the original cell lineage of the K562 cell line. This possibility remains to be tested by analysis of the ξ globin gene initiations in K562 cells. We have not carried out detailed transcription study of the α1 globin upstream RNA in other human non-erythroid cell lines, except for a Hela cell line where no α globin RNA was detected (data not shown).

Subcellular Location of the Upstream Transcript

One interesting result which emerged from our study is the efficient transport of the α1 globin upstream transcript from nucleus to the cytoplasm of transfected COS-7 cells. A previous study of the subcellular location of RNA transcripts produced from human β globin gene transfected into COS cells showed that the only RNA transcripts found in the cytoplasm were those with 5' termini at or downstream from the canonical cap site (13). All other studies of globin transcripts in

human tissues have been carried out on total cellular RNA, and thus the subcellular distribution of RNA species were not determined (9-12). Our results clearly contradict the hypothesis that RNA transcripts with 5' termini mapped upstream from the normal cap site are not transported to the cytoplasm (13). We do not know whether this difference in subcellular location of the α- and β-globin upstream transcripts results from the difference in their primary sequence, secondary structure, or post-transcriptional modification of the RNA.

Origin and Possible Function(s) of the Upstream Transcripts

Despite the seemingly universal occurrence of upstream initiation sites of different eukaryotic genes (1-15, this study), relatively little is known about their function. The hypothesis that upstream initiation sites may serve as RNA polymerase II entry sites is plausible (14) and awaits further proof. The presence of cap structure on some of these upstream transcripts(for example, see ref. 9) and their transport to the cytoplasm (this study) suggest that they may be translated. The upstream initiated RNAs of SV40 early region have been shown to contribute to the formation of T-antigen late in the infection cycle (25).

The mechanism responsible for the initiation of upstream transcripts is also not clear. In the case of the SV40 early region, Buchman et al. (26) have demonstrated that the upstream initiation is regulated by DNA replication. Early during infection, transcription is initiated from sites downstream from the replication origin (the canonical sites). Binding of T antigen in the early promoter region, which occurs late during the infection cycle, then represses transcription from the canonical sites. Concurrently, transcriptional initiation from the upstream sites is activated by DNA replication. Furthermore, the initiation from the canonical sites but not the upstream sites is highly dependent on the presence of enhancer sequence in cis. This independent regulation of upstream and canonical initiation by enhancer sequence and by DNA replication has also been observed for the human embryonic ε-globin gene (14).

In the cases of β-globin gene family, both RNA polymerases II and III seem to play a role. For instance, some upstream transcripts from the ε globin gene are capped, and thus are synthesized by RNA polymerase II (9). Examination of the nucleotide sequences flanking the upstream initiation sites has also revealed some elements homologous to the eukaryotic pol II promoters (for example, see ref 7, 10, 12). However,

this is not always true. Furthermore, because promoter-like sequences are relatively common in the 5' flanking regions of the β-like globin genes, and they are not always associated with initiation of transcription, the possibility that weak polymerase II-promoters are responsible for these transcripts must also be answered. There is at least one upstream transcript (-176, Table 1) from the adult β-globin gene that seems to be synthesized by RNA polymerase III (11).

Several lines of evidence suggest that the upstream RNA from the α1 globin gene is initiated by polymerase II instead of polymerase III. First, its initiation *in vitro* is inhibited by α-amanitin at a concentration of 2 μg/ml (Fig. 5B). Secondly, analysis of the DNA sequences from bases -250 to +50 does not reveal any region homologous to the consensus sequence of polymerase III-promoter. Thirdly, detailed studies on RNA polymerase III-dependent transcription of the human adult α2-α1 globin gene region in cell free extracts did not reveal any transcript initiated immediately upstream from the α1 globin gene (23, 27, 28). As shown in Fig. 1B, the putative initiation site (-45) of the α1 globin upstream RNA lies in between the CCAAT box (-71 to -67) and the ATA box (-28 to -26) which are required for α1 globin gene transcription *in vivo* (18). Transcriptional study of the α1 globin promoter containing different point mutations should reveal whether initiation events at the upstream and canonical sites are tightly coupled, and whether they are controlled by the same promoter elements.

Finally, it is interesting to point out that the putative 5' end of the -45 transcript maps in a region that contains many copies of a GGGC segment in both orientations (Fig. 1B). This is similar to a sequence in the SV40 21 bp repeat, and in the 5' flanking region of the dihydrofolate reductase gene that appears to behave as a bi-directional promoter (29). In particular, the hexanucleotide sequence pointed out by Farnham *et al.* (29), GGGCGG is present on the anti-sense strand at -61 to -56 (Fig. 1B) and regions further upstream (sequences not shown). The detection of the α1-upstream transcript suggests that some of these GC-rich sequences may constitute a type of RNA polymerase II promoter. The possibility of "antisense" transcription in the α1-globin promoter region is also worthy of future investigation.

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