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Initiation of transcription of the erythroid promoter of the porphobilinogen deaminase gene is regulated by a *cis*-acting sequence around the cap site

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Received September 6, 1990; Accepted October 8, 1990

ABSTRACT

Although the erythroid-specific promoter of human porphobilinogen deaminase [PBGD] gene has no TATA box, transcription is initiated at a single nucleotide. Using 5′ and 3′ deletions and point mutations, we have identified an element, located around the initiation site, which is necessary and sufficient for '*in vitro*' accurate initiation of transcription. This 15 bp element extends 1 bp 5′ and 14 bp 3′ from the initiation site. It is composed of two regions, a proximal region centred on the cap site and a distal region which bears homology with the TdT initiator element. We show that a nuclear factor, present both in erythroid and non erythroid cells, binds the distal PBGD initiator element. Lack of heat inactivation suggests that initiation of transcription mediated by this element is not TFIID dependent. By transfection into erythroid cells, we also show that the proximal PBGD initiator element is essential for the selection of the initiation site but not for the regulation of transcription of the PBGD erythroid promoter during erythroid differentiation.

INTRODUCTION

Efficiency and accuracy of transcription by RNA polymerase II seem to be determined by two classes of trans-acting factors. One class includes factors which recognize sequences located 30 bp or more, upstream or downstream, from the cap site and regulate the rate of RNA polymerase II initiation of transcription. Most of the trans-acting factors characterized until now belong to this class and are gene-specific transcriptional activators since deletions or mutations of their binding sites result in reduction of transcriptional activity but do not alter the site of initiation [for review see **(1)**]. The mRNA start site seems to be selected by a second class of factors and needs, near the initiation site, the assembly of a multicomponent complex containing a set of general transcription factors which interacts directly with RNA polymerase II to ensure accurate initiation of transcription [for review see **(2)** and **(3)**]. Among these general transcription factors, only TFIID seems to be sequence specific by binding to an A + T rich region called TATA box **(4)**. Mutations on this element result in a significant decrease in the level of transcription together with a slight alteration in the site of transcription initiation **(5)**.

Many cellular genes do not contain obvious TATA box at −30 and still initiate transcription at only one start site. Most of those genes are highly regulated during differentiation and/or development but are expressed at low level. No obvious consensus sequence involved in the choice of the initiation site of those genes has been defined but recently, the region containing the start of initiation of transcription of three mammalian genes has been demonstrated to locate the initiation site **(6−9)**.

During erythroid differentiation, a set of specific genes are coregulated at the transcriptional level. Among these genes, the globin genes possess a TATA box but most of the cloned erythroid specific genes, like the human glycophorin A **(10)**, the mouse erythroid 5-aminolevulinate synthase **(11)** and the mouse erythropoietin receptor [our unpublished result] do not possess a TATA box around −30 and still initiate transcription at a single nucleotide. We have focused our work on the regulation of the expression of the human gene coding for porphobilinogen deaminase [PBGD], the third enzyme of the haem biosynthetic pathway. This gene has two overlapping transcription units, each with its own promoter **(12)**. The upstream promoter is active in all cell types tested whereas the downstream one is erythroid specific. We have shown that the erythroid transcription unit was initiated at a single nucleotide although no TATA box was present around −30 in this erythroid promoter. By transfection in murine erythroleukaemia (MEL) cells, we have demonstrated previously that a cis-acting sequence, located between −80 and −50, was necessary for accurate initiation of transcription **(13)**. This sequence contains a binding site for NF-E1, the major DNA-binding protein of the erythroid lineage **(14)**.

In this paper, we examine the cis and trans-acting elements involved in the accurate transcription initiation of the erythroid PBGD transcription unit and define an element located around the cap site and dominant both in vitro and in vivo for the determination of the site of initiation of transcription.

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MATERIALS AND METHODS

Cells and nuclear extracts

An APRT$^-$ MEL cell line **(15)** was maintained in Dulbecco's modified Eagle's medium supplemented with 15% horse serum and 50 μg/ml diaminopurine. K562 and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Nuclear extracts from K562 or HeLa cells were prepared as described **(16)** and also from mouse liver or erythropoietic spleen from an anaemic mouse **(17)**.

Plasmid DNAs

Subcloning and plasmid DNA manipulations were performed **(18)**. For site-directed mutagenesis, the −714 to +78 and the −50 to +78 regions of the human PBGD erythroid promoter **(13)** were subcloned into phage M13 mp18 and used as template for mutagenesis by using the Amersham 'oligonucleotide-directed in vitro mutagenesis system'. The mutants were sequenced and the mutated promoters were then subcloned into pBL-CAT3 **(19)**. 5′ and 3′ deletion mutants were generated by using complementary oligonucleotides as indicated in the text and figures. After annealing, these oligonucleotides produced 5′ HindIII and 3′ BamHI ends. The double stranded oligonucleotides were ligated into pBL-CAT3 and the ligation mixture was used to transform bacteria. DNA was prepared from individual colonies and sequenced. The PBGD minigene was obtained by ligation of an EcoRI-BamHI fragment containing 714 bp upstream from the erythroid initiation site and the first 78 bp of the erythroid-specific exon to a BamHI-XbaI fragment isolated from the human PBGD cDNA and an XbaI-EcoRI fragment containing the last intron and exon as well as 300 bp of the 3′ region of the human PBGD gene [for details see **(12, 20)**].

In vitro transcription experiments

Templates for *'in vitro'* transcription were prepared by cleaving the individual plasmid by BamHI when the plasmid contained 78 bp downstream from the initiation site or EcoRI when the 3′ deletion plasmids were studied. Transcription reactions were performed in a total volume of 25 μl containing 40−80 μg/ml of DNA and 2−4 mg/ml nuclear protein extract in a buffer containing 12 mM Hepes pH = 7.9, 12% v/v glycerol, 0.3 mM dithiothreitol (DTT), 0.12 mM E.D.T.A., 60 mM KCl, 12 mM $MgCl_2$, 600 μM XTP and 30 units of RNAsin [Promega

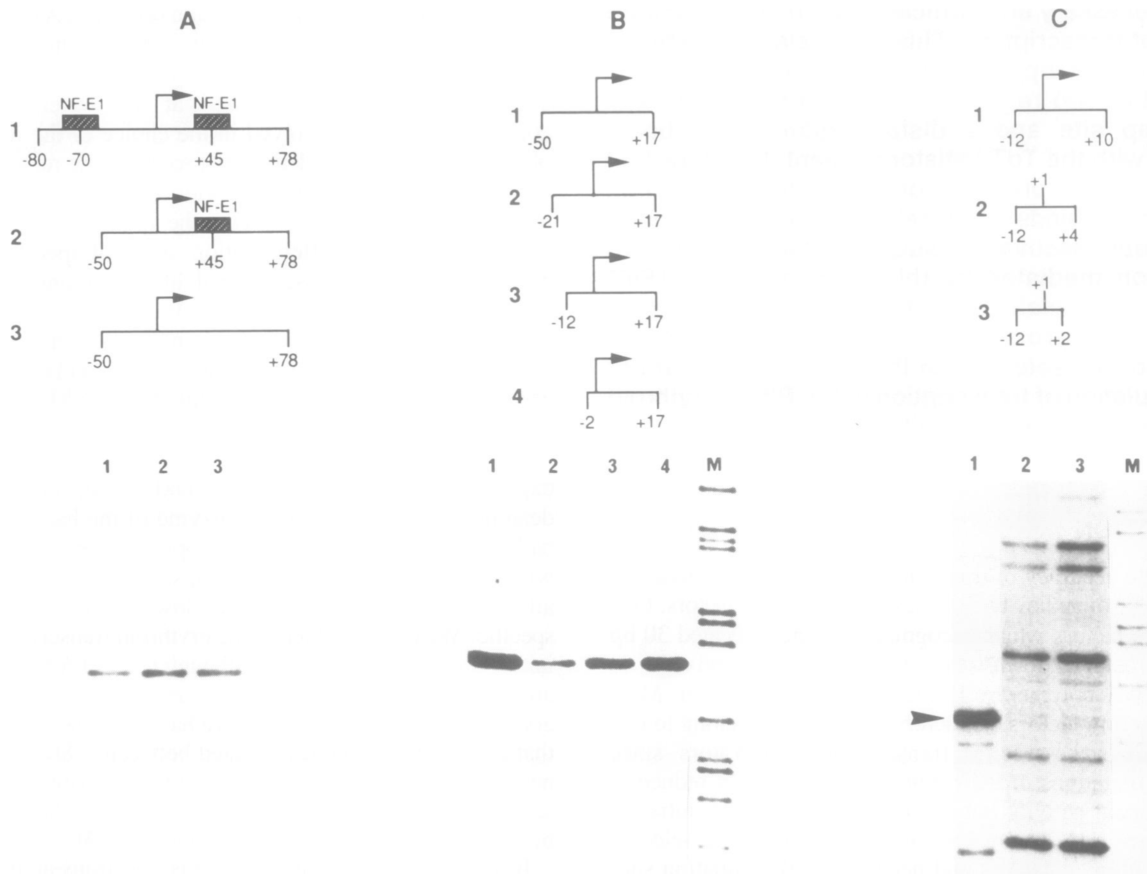

Figure 1. Mapping of the initiator element of the PBGD erythroid promoter. Plasmids containing DNA sequences shown above the autoradiographs were linearized at EcoRI or BamHI and their transcriptional activity was analyzed. The RNAs produced were mapped by primer extension using a chloramphenicol acetyl transferase [CAT] or a PBGD specific oligonucleotide primer [see Materials and Methods]. **(A)** The −80/+78 DNA fragment of the PBGD erythroid promoter [lane 1], the −50/+78 DNA fragment which did not contain the −70 NF-E1 binding site [lane 2] and the −50/+78 DNA fragment mutated on the +45 NF-E1 binding site [lane 3] were transcribed with equal efficiencies and promoted accurate initiation. The arrow indicates the extended product. **(B)** 5′ deletion mutants with 3′ end fixed at nucleotide +17 were analyzed. All mutants were transcribed and initiated mRNA at the same site than the −50/+17 PBGD DNA fragment [compare lane 1 (−50/+17) and the other lanes]. Marker [lane M] was a G reaction of a known sequence. **(C)** 3′ deletion mutants with 5′ end fixed at nucleotide −12 were analyzed. The −12/+10 mutant [lane 1] was efficiently and accurately transcribed but the −12/+4 (lane 2) and the −12/+2 (lane 3) mutants gave rise to weak and multiple initiation sites. Marker [lane M] was a G reaction and the arrow indicated the extended product obtained when RNAs were accurately initiated.

Biotec]. Following incubation at 30°C for 45 minutes, RNA was prepared for primer extension as described (17).

Transfections into MEL cells

Protoplast fusions were carried out (21) and RNA was isolated before and after hexamethylene bisacetamide (HMBA) (5 mM final concentration) induced MEL cell differentiation.

Protein-DNA interactions

Gel retardation assays were performed with K562 or HeLa nuclear extracts as described (22) except that no $MgCl_2$ was added.

Methylation interference assays

The labelled probe was partially methylated and after protein-DNA interactions, the free and bound oligonucleotides were resolved, excised and electroeluted from a native gel. The nucleic acids were NaOH treated and the radioactive samples were separated on a 12% sequencing gel (23)

RNA analysis

Primer extension analysis of mRNAs utilized either a PBGD specific oligonucleotide primer hybridizing 67 nucleotides from the transcription start site [for details see (12)] or a Chloramphenicol Acetyl Transferase (CAT) specific oligonucleotide primer [5′ TCCTTAGCTCCTGAA AATCT-CGCC 3′] hybridizing 47 nucleotides from the BamHI site where the PBGD constructs were linked to the CAT sequence. Hybridization and reverse transcription assays were done as previously described (24).

RESULTS

Deletion analysis of the human PBGD erythroid promoter defined a minimal region involved in determination of the initiation site

We have previously shown that a −80/+78 DNA fragment of the human erythroid PBGD promoter was sufficient to ensure correct initiation after transfection in MEL cell (13). This fragment contains two binding sites for NF-E1, one at −70 and one at +45 and no TATA box around −30. As the −50/+78 DNA fragment did not ensure correct initiation '*in vivo*', the −70 NF-E1 binding site was a good candidate for a cis-acting sequence involved in the determination of the site of transcription initiation of the erythroid PBGD mRNA. Using *in vitro* transcription assays, we found that the −80/+78 DNA fragment was able to direct accurate and specific initiation of transcription with mouse adult liver nuclear extract or with a cell line nuclear extract complemented with mouse anaemic spleen nuclear extract [data not shown]. We used this latter protocol [a human erythroleukaemic cell line (K562) complemented with anaemic spleen nuclear extract] to study the PBGD erythroid promoter. In this '*in vitro*' transcription assay, the −50/+78 DNA fragment was as effective as the −80/+78 DNA fragment [Figure 1A, lanes 1 and 2] and −50/+78 DNA fragment mutated in the +45 NF-E1 binding site was also able to ensure correct initiation of transcription [Figure 1A, lane 3]. This transcription was sensitive to 2 μg/ml α-amanitin and linear or supercoiled templates were equally active [data not shown]. We generated a set of 5′ deletion mutants which contain various lengths of the PBGD erythroid promoter and 17 bp 3′ from the initiation site [Figure 1B].

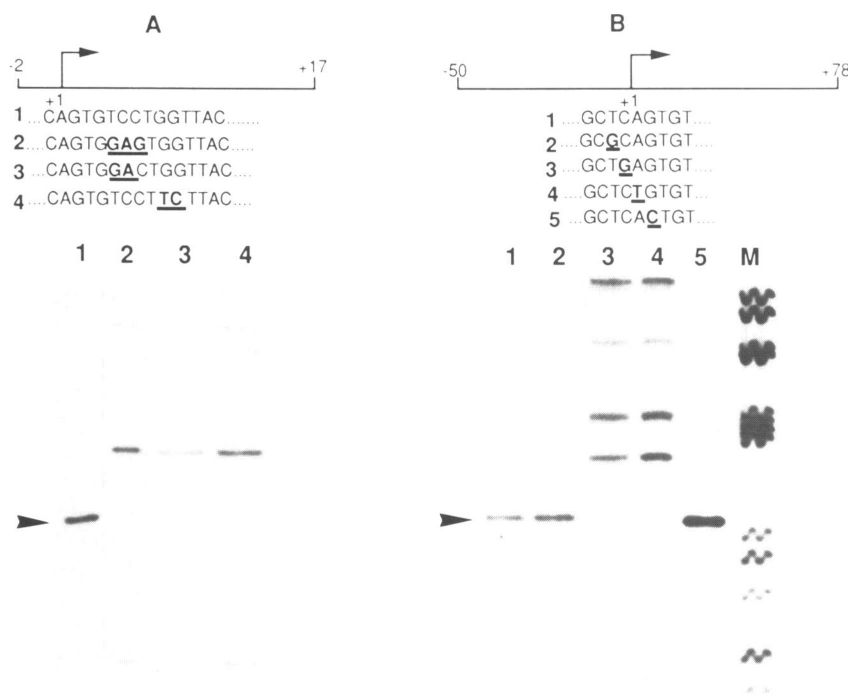

Figure 2. Two regions around the PBGD initiation site are required to position the transcription initiation. (A) The −2/+17 DNA fragment of the PBGD erythroid promoter [lane 1] and mutants which contained the mutations indicated were transcribed '*in vitro*' [lanes 2, 3 and 4]. The arrow indicates the extended product obtained when RNAs were accurately initiated. (B) Four mutants of the −50/+78 DNA fragment were obtained by site-directed mutagenesis. Each had a transversion on −2, −1, +1 or +2. Mutants −2 or +2 promoted accurate initiation of transcription (lanes 2 and 5) whereas mutants −1 and +1 produced non accurate initiation of transcription (lanes 3 and 4). Lane 1 was the product obtained after transcription of the −50/+78 DNA fragment. Marker [lane M] was a G reaction. The arrow indicates the extended product obtained when RNAs were accurately initiated.

Analysis of their transcriptional activity showed that all the constructs including the −2/+17 which had only two nucleotides upstream from the cap site, were as effective as the −50/+17 DNA fragment in ensuring accurate initiation of transcription [Figure 1B, compare lanes 1 and 4]. Similar results were obtained when those constructs were inserted into a different vector [Bluescript KSII+] indicating that the PBGD initiator element was able, by itself, to promote accurate and efficient transcription (data not shown). We then generated a set of 3′ deletion mutants and analyzed their transcriptional activities. The −12/+10 construct directed accurate initiation of transcription [Figure 1C, lane 1] whereas the −12/+4 and −12/+2 mutants displayed levels of transcription that were about 10% of the −12/+10 construct and directed heterogeneous initiation of transcription that lay mainly within the vector we used [Figure 1C, lanes 2 and 3].

Point mutations on the minimal promoter of the erythroid PBGD gene defined two regions involved in the determination of the site of transcription

In the element previously characterized, a sequence TCC-TGGTTAC, between +5 and +14 seemed essential for efficient initiation, of transcription and shared homology with the +2 → +11 region of the murine TdT gene [TTCTGGAGAC] which is part of the TdT initiator element. The −12/+10 construct, which was efficiently and accurately transcribed, also had a similar sequence, TTCTGGATCC, between +5 and +14, since we cloned the 3′ deletion mutants at a BamHI site. We mutated the TCCTGGTTAC sequence and analyzed three mutants. One with a TCCTGG → GAGTGG mutation [construct 2], one with a TCCTGG → GACTGG mutation [construct 3] and one with a TCCTGG → TCCTTC mutation [construct 4]. As for the −12/+4 and −12/+2 mutants, these three mutants failed to drive accurate initiation, displayed very low levels of transcription and multiple initiations of transcription within the vector were observed [Figure 2A, lanes 2, 3 and 4].

To determine if another element of the PBGD initiator element was involved in the '*in vitro*' accurate initiation of the transcription of the PBGD erythroid promoter, we performed site-directed mutagenesis on nucleotides −2, −1, +1 and +2 of the −50/+78 DNA fragment and analyzed the '*in vitro*' transcriptional activity of these four mutants. As shown in Figure 2B, transversions on the −1 and +1 nucleotides impaired the accurate initiation of transcription [Figure 2A, lanes 3 and 4] whereas transversions at positions −2 and +2 did not impair initiation of transcription [Figure 2A, lanes 2 and 5].

Initiation mediated by the initiator element is not TFIID dependent

It has been shown recently that heat treatment of nuclear cell extract results in inactivation of TFIID dependent initiation of transcription (**25**). Using the human uroporphyrinogen decarboxylase [URO-D] promoter (**26**) as a control [the initiation of transcription driven by this promoter is dependent of its TATA box (our unpublished data)], we asked whether the initiation of transcription mediated by the PBGD initiator element was TFIID dependent. Nuclear cell extracts were heated for 15 minutes at 47°C and immediately used for '*in vitro*' transcription. As shown in Figure 3, the initiation of transcription of the URO-D gene was completely abolished after this treatment whereas the initiation of transcription of the PBGD gene was unaffected.

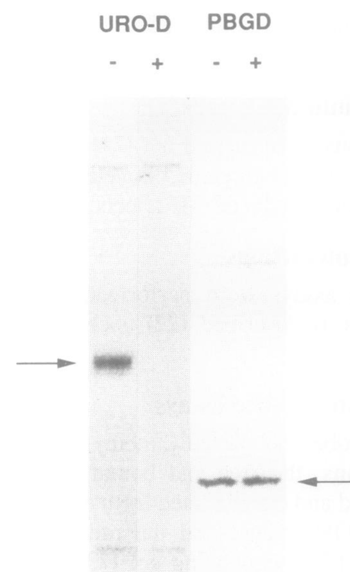

Figure 3. Effect of heat treatment on the PBGD initiator element mediated initiation of transcription. Nuclear extracts were split and half was treated for 15 minutes at 47°C. *In vitro* transcription with UROD or PBGD templates was performed and RNAs were mapped by primer extension. The arrows indicate the mRNAs start sites. −: without heat inactivation, +: after heat inactivation.

A nuclear factor, present in erythroid and non erythroid cells, interacts with the PBGD initiator element

To identify proteins interacting with the PBGD initiator element, various oligonucleotides were used in gel retardation assays. An oligonucleotide [oligo A] containing 2 bp upstream and 15 bp downstream from the initiation site bound a protein present in erythroid and non erythroid cells [Figure 4A, lanes 1 and 8] and competitions showed that this binding was specific [Figure 4A, lanes 2 and 3]. We then analyzed three oligonucleotides containing mutations that abolished accurate initiation of transcription. Two of them [oligo B (lane 4) or oligo C (lane 5)] contained mutations in the TCCTGGTTAC box and could not inhibit the binding of the protein to oligo A. In contrast, an oligonucleotide [oligo D] bearing a C → G transversion at −1 efficiently competed with this binding [Figure 4A, lane 6]. Finally, competition with a 17 mer oligonucleotide [oligo E] containing the +2 → +11 region of the murine terminal transferase initiator element was ineffective [Figure 4A, lane 7] suggesting a different affinity of this nuclear factor for the PBGD or TdT initiator elements in the conditions we used.

A methylation interference assay was performed to determine the binding site of this factor more accurately. As shown in Figure 4B, the protein interacted strongly with the G located in both strands of the GGTTAC sequence but also with most of the guanines located between this sequence and the cap site.

All these data suggested that the CCTGGNNAC sequence was necessary but not sufficient for the binding of this protein, and that non accurate initiation of transcription by the −1 mutant was not accounted for by lack of binding of this protein.

In vivo, the proximal initiator element is dominant over all the other cis-acting sequences of the PBGD erythroid promoter for determination of the initiation site

The initiator element defined above was necessary and sufficient to direct accurate *in vitro* initiation and we next analyzed the

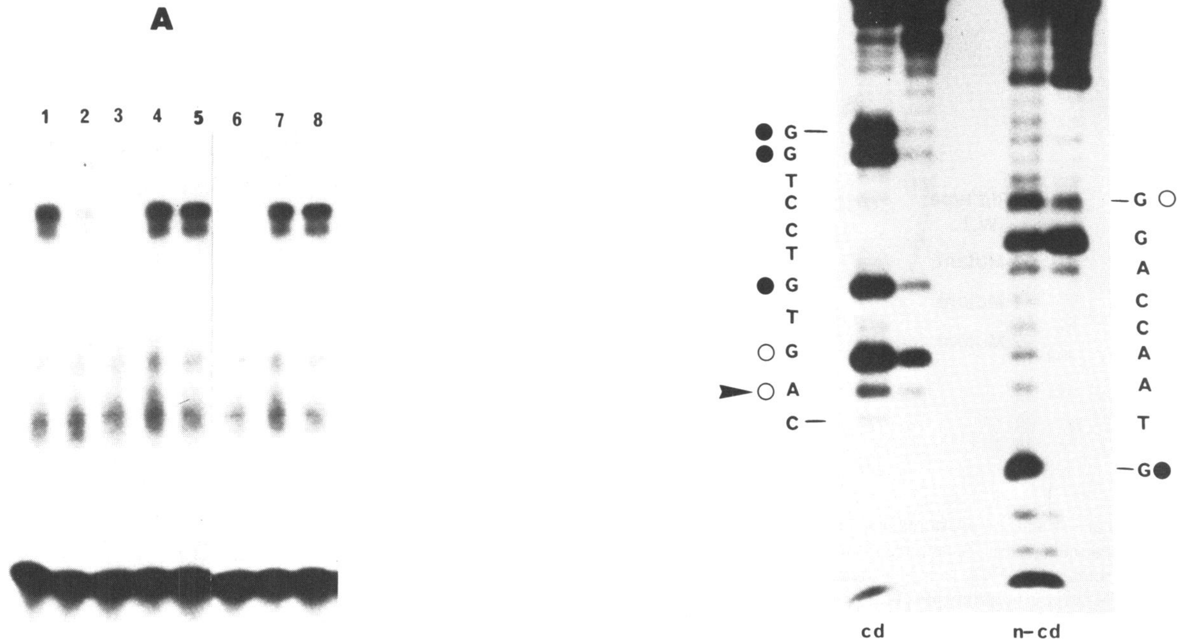

Figure 4. Characterization of a nuclear factor that bound the PBGD initiator element. All the oligonucleotides were 17 bases double stranded DNA and 5 bases single-stranded DNA with 2 bases 5′ and 3 bases 3′ from the double stranded region. The indicated length was that of the double stranded DNA. **(A)** Gel mobility shift assay: 5 μg of nuclear extract from HeLa [lane 1] or K562 [lane 8] were incubated with a 17 bases oligonucleotide containing 2 bp upstream and 15 bp downstream the PBGD erythroid mRNA start site [oligo A]. Lanes 2 and 3 were the K562 mobility shift assay in presence of 5 (lane 2) and 100 (lane 3) fold excess of cold oligonucleotide A. Lanes 4 and 5 were competitions with 100 fold excess of two PBGD initiator oligonucleotides [oligo B and oligo C] mutated on the TCCTGGTTA-C box. Lane 6 was a competition with the 17 bases oligonucleotide mutated on −1 [(C → G)] [oligo D] and lane 7 was a competition with a 17 mer oligonucleotide containing the TdT initiator element [oligo E]. **(B)** Methylation interference experiment on both strands of the PBGD initiator element oligonucleotide. The retarded as well as the free DNA bands were excised from a native gel and treated as indicated in Materials and Methods. The sequence of both strands is indicated; open and closed circles indicate weak and strong interference. The site of initiation is indicated by an arrow. F = free DNA. B = bound DNA. cd = coding strand. n-cd = non coding strand.

interaction between the cis-acting sequences located upstream from the initiator element and the initiator element *in vivo*. Since the TdT initiator element has been partially characterized by transfection, we focused our experiments on the proximal PBGD initiator element.

We have previously shown that the −714/+78 DNA fragment from the human PBGD erythroid promoter was sufficient for correct initiation and regulation of a linked reporter gene when introduced into MEL cells **(24)**. Using site-directed mutagenesis, we constructed three mutants of this −714/+78 DNA fragment, one on −1, one on +1 and one on +2 [Figure 5A]. We then linked the −1, +1 and +2 mutants, together with the wild type −714/+78 DNA fragment, to a PBGD minigene described in Material and Methods. These constructs were inserted into a plasmid containing the hamster gene for adenine phosphoribosyltransferase [APRT] and used to transform competent *Escherichia coli*. After protoplast fusion with MEL $APRT^-$ cells, several pools of more than 100 independent $APRT^+$ clones were obtained. Differentiation was induced with HMBA and human PBGD mRNA was probed before and after HMBA induction by primer extension. As shown in Figure 5B, the −1 and +1 mutants produced a series of initiations dispersed over at least 20 bases, were weakly transcribed but correctly regulated (their transcriptional activities were enhanced after induction) whereas the wild type promoter and the +2 construct were correctly initiated and regulated.

DISCUSSION

In *Escherichia coli*, sequences between −10 and −35 determine the start site of transcription **(27)** whereas in yeast, the initiation region itself is involved in the accurate initiation of transcription **(28)**. In many highly expressed, tissue-specific genes, the TATA box seems to be the dominant element in determining the start site of transcription **(29)**. In contrast, studies on the promoter of mouse or rabbit β-globin genes have indicated that deletions or mutations within the TATA box lead to a drastic decrease in the level of transcription but very little heterogeneity in the start site of the few transcripts made **(5, 30)** suggesting that sequences apart from the TATA box could ensure weak but accurate initiation of transcription. In this paper, we present evidence that the initiation of transcription of a highly regulated transcription

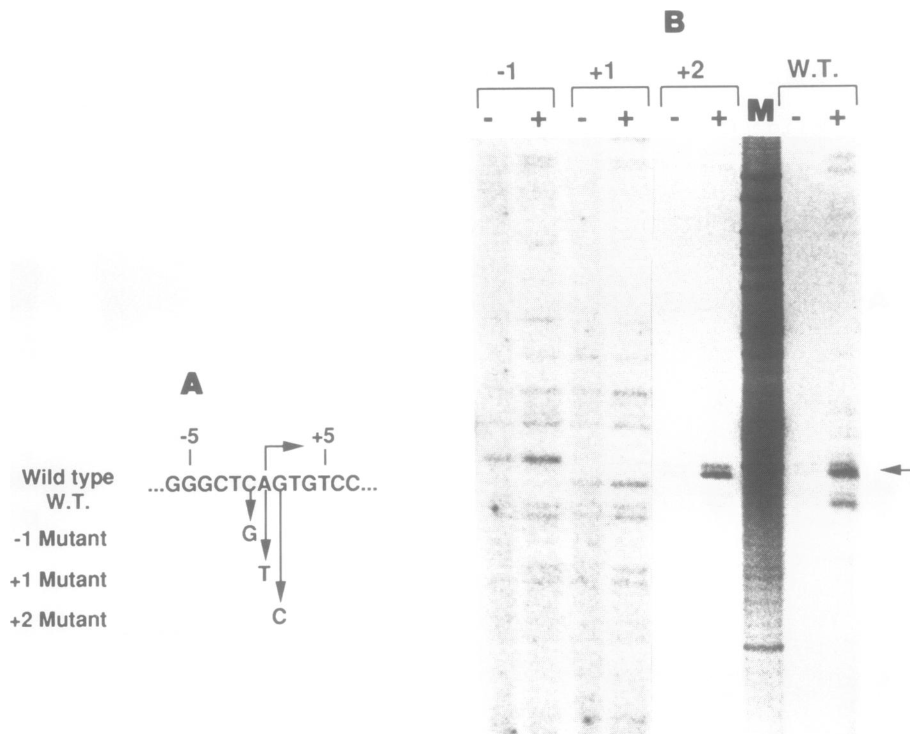

Figure 5. Mutations at −1 and +1 are dominant '*in vivo*' over the other cis-acting sequences of the PBGD erythroid promoter for accurate initiation of transcription. **(A)** Using site-directed mutagenesis, three mutants of the PBGD erythroid promoter were generated. W.T.: wild-type −714/+78 DNA fragment of the PBGD erythroid promoter. −1: −1 mutant [C → G]; +1: +1 mutant (A → T) and +2: +2 mutant [G → C]. **(B)** The three mutants (−1, +1 and +2) and the −714/+78 DNA fragment of the PBGD erythroid promoter (W.T.) were transfected into MEL cells and pools of stable transformants were induced by HMBA for 96 hours. RNAs were extracted before (−) or after (+) HMBA induction and mapped by primer extension. Marker [lane M] was a G reaction. Since the −1 and +1 mutants were weakly transcribed, two autoradiographies of the gel were performed, one overnight [W.T. and +2] and one for 4 days [−1 and +1].

unit can be completely independent of a TATA box element. Together with previous results **(13)** our data indicate that an interaction between a tissue-specific factor [NF-E1] and a general transcription factor that binds sequences around the cap site might mediate the accurate initiation of transcription of the human erythroid PBGD gene '*in vivo*'.

The mouse PBGD gene has been cloned recently and analysis of its erythroid transcription unit has shown that it is initiated 34 bp upstream from its human counterpart **(31)**. Inspection of the sequence of the mouse PBGD erythroid promoter region revealed two potential 'initiator' sequences, one at the same location as the human PBGD initiator element and another one, 34 bp upstream [Figure 6]. The mouse erythroid PBGD mRNA start site occurred only within this upstream initiator element. Examination of the mouse sequence around the human initiation site [+36 in the mouse sequence] revealed three mutations in the CTGGNNAC sequence and no mutation around the cap site while analysis of the human sequence around the mouse initiation site [−32 in the human sequence] revealed a C → G transversion at −33 and no major mutation in the distal sequence of the initiator element. This observation supports the function of the two boxes of the PBGD initiator element in selection of the initiation of transcription.

Recent studies have demonstrated that different mechanisms can promote accurate initiation of transcription of cellular genes that lack TATA box and are not GC rich. The mRNA start sites of two housekeeping genes, the murine dihydrofolate reductase [DHFR] and the murine ribosomal protein rpS16, are dependent on initiator elements which do not display any structural

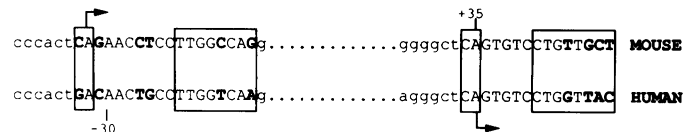

Figure 6. Sequence comparison of the mouse and human PBGD erythroid promoter. Initiation of transcription of the mouse and human PBGD erythroid mRNAs is indicated by an arrow. The first nucleotide of each promoter is numbered −1 and the first nucleotide of the first exon is numbered +1. The putative initiator elements are boxed and the nucleotides which differ are indicated in bold-faced letters.

homology. These initiator elements need additional cis-acting sequences *in vitro* and *in vivo* to promote efficient transcription **(7, 8)**. These results are in contrast to our findings that the PBGD initiator element was able, by itself, to promote efficient accurate initiation of transcription *in vitro*. This property is shared with the murine terminal deoxynucleotidyltransferase [TdT] initiator element which can promote accurate and efficient transcription *in vitro* and *in vivo* **(6)**. Thus, initiator elements of tissue specific genes displayed properties different from the housekeeping genes initiator elements.

The PBGD initiator element alone cannot promote accurate transcription *in vivo* but point mutations within this element abolished accurate initiation of transcription of a −714/+78 DNA fragment containing the erythroid PBGD promoter and stably introduced into MEL cells. As all the initiation sites used by these mutated promoters were upregulated during HMBA induced

MEL cell differentiation, we would like to suggest that the main action of the PBGD initiator element is to select the correct start site of transcription of the erythroid PBGD mRNA. This result is in contrast to that observed with the TdT initiator element which drives efficient and correct initiation by itself, and suggests that these two cis-acting elements act via different mechanisms. This hypothesis is supported by the existence of a nuclear factor binding the PBGD initiator element and probably not the TdT initiator element. Indeed, as the erythroid PBGD transcription unit is embedded in an open chromatin domain both in erythroid and non erythroid cells (**24** and our unpublished data), the control of initiation start of the PBGD erythroid mRNA has to be highly regulated and the PBGD initiator element might participate in this regulation.

ACKNOWLEDGEMENTS

We thank Professor J.Rosa for support and encouragement, C. Chabret for technical assistance and R.Quintel for assistance in the preparation of the manuscript. We thank K.Mayne for revision of the manuscript. We also thank Dr A.Schechter for receiving D.Beaupain in his laboratory and E.Quattrone for her help in many steps of this work. This work was supported by grants from Institut National de la Santé et de la Recherche Médicale (U.91), Centre National de la Recherche Scientifique (AIP 1985 06931), and the Association pour la Recherche contre le Cancer [Grant 6416].

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