In Vitro Transcription From the Human αγ-Globin Gene Promoter

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We report enhanced transcription from the human αγ-globin gene promoter in nuclear extracts (NE) of erythroleukemia (K562) cells compared with that in HeLa NE. We do not observe differences in transcription levels in the two extracts with nonglobin promoter templates. Our findings, indicating preferential recognition of the globin gene promoter by nuclear factors in K562 cells, are consistent with results of studies previously reported by ourselves and others. A novel finding described here is that the addition of a double-stranded octamer motif oligonucleotide to K562 NE increases the level of transcription from the αγ-globin gene promoter, suggesting a potential role for an octamer motif-binding factor in the repression of αγ-globin gene transcription. A cosmid construct containing extensive human α- and β-globin gene promoter and structural sequences as well as upstream control sequences also exhibits higher levels of globin gene transcription in K562 NE than in HeLa NE. Our demonstration of the feasibility of efficient, globin promoter-specific in vitro transcription of this complex template offers a novel approach for the systematic analysis of the effects of putative regulatory factors on globin gene expression in vitro in the context of a genetic environment approximating that found in vivo.

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MATERIALS AND METHODS

Cell lines and nuclear extracts (NE). The human erythroleukemia cell line K562 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, initially in tissue culture flasks and subsequently in spinner flasks. HeLa cells were similarly grown, except in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. NE were prepared as described, except for the addition of leupeptin, pepstatin, and aprotonin (1 μg/mL each) to all solutions to inhibit proteolyis. Protein concentration of NE was determined by the Bradford method using a kit from Bio-Rad (Heracles, CA) and bovine gamma globulin as a standard. Extracts were stored in small aliquots at −15°C.

DNA templates used for in vitro transcription. Plasmids pγneo, pRSVneo, and pMLP were used as templates for in vitro transcription reactions. Plasmid pγneo is a derivative of pSV2 and was constructed as described. The pγneo plasmid contains 1.3 kb of human α-globin gene sequence extending from the 5′ flank into the 3′ untranslated region linked to the neomycin resistance (neoR) gene. Plasmid pRSVneo, another pSV2 derivative, was the gift of Silvana Obici; it contains the Rous sarcoma virus long terminal repeat (LTR) promoter and was constructed as described. Plasmid pMLP was the gift of Michael Reach and Hamish Young (Columbia University, New York, NY) and was constructed by insertion of a 459-bp HindIII-XhoI fragment containing the major late promoter (MLP) of adenovirus 2 into vector pSP64. Cosmid µLCRγχβδ was also used as a template in some experiments. The cosmid, a gift of T. Enver (Institute for Cancer Research, London, UK), was constructed as described. It contains functional but abbreviated sequences from the β-globin locus control region (LCR) linked to 29-kb containing sequences extending from upstream of the γ-globin gene to a Kpn I site downstream of the β-globin gene.

In vitro transcription reactions. Transcription reactions were performed under conditions as described by Dignam et al.

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concentrations in the transcription reaction mixtures were 12 mmol/L HEPES, pH 7.9, 12% glycerol, 0.3 mmol/L dithiothreitol (DTT), 0.12 mmol/L EDTA, 60 mmol/L KCl, and 12 mmol/L MgCl₂. Modifications were all four ribonucleoside triphosphates (rNTPs) were unlabeled and present at a concentration of 600 mmol/L each and reaction volumes ranged from 50 to 125 µL. Incubation was at 30°C for 1 hour. Other conditions are indicated in the figure legends. Processing of in vitro-transcribed RNA was as described, except that ethanol precipitation was in 2 mol/L ammonium acetate, pH 5.5. Annealing and primer extension were as described, except that annealing was for 15 minutes at 65°C and gradual cooling was allowed to occur to 42°C. Primer extension reactions were at deoxynucleoside triphosphate (dNTP) concentrations of 3.3 mmol/L and were incubated at 42°C to 45°C. In vitro-synthesized transcripts of pyneo, pRSVneo, pMLP, and the cosmid construct were hybridized to oligodeoxynucleotide primers complementary to their respective RNAs (5'-CCTCATCTGTCTCTCGATC3', complementary to neo sequences of pyneo; 5'-AGCTGGCATTCCGTTTCGCTG3', complementary to neo sequences of pRSVneo; 5'-CGACTGGCCTTCGGCAGGCAGC3', complementary to pMLP sequences; 5'-CTCCCTCTTGATTAGCTCCATGCGCTCTG3', complementary to human γ-globin sequences; and 5'-CCACAGGGCAGTAACGGCAGA3', complementary to human β-globin sequences). The primers were 5'-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Ten to 25 fmol of primer was used per reaction to ensure primer excess.

Transcription reactions with competitor oligonucleotides. All reactions contained double-stranded poly(dllC), which reduced the effects of nonspecific DNA binding proteins and added greater consistency between experiments. For oligomer competition experiments, NE were incubated with poly(dllC) alone or with an equivalent total weight of poly(dllC) plus double-stranded oligomer for 15 minutes on ice followed by a 15-minute room temperature incubation. Transcription reactions were then initiated by the addition of template and rNTPs. Ethanol-precipitated reaction products were washed in 70% ethanol, resuspended in formamide loading dye, and electrophoresed on an 8% polyacrylamide, 7 mol/L urea sequencing gel. Gels were dried and exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY) at -70°C with an intensifying screen. Double-stranded oligomers were purchased from Genosys (Woodlands, TX).

RESULTS

In vitro transcription from the γ-globin gene promoter with K562 and HeLa NE. Plasmid pyneo, containing the human γ-globin gene promoter linked to a neomycin resistance gene, was transcribed in vitro. The products resulting from primer extension of transcripts synthesized from the pyneo template in uninduced K562 and HeLa NE were appropriately sized (ca. 72 bases), indicating transcription initiation at the 5' cap of the γ-globin gene, as shown in Fig 1. Transcription levels increased as the total NE concentration was increased both in reactions containing HeLa NE supplemented with K562 NE (lanes 1 and 2) and in unsupplemented HeLa NE reactions (lanes 3 and 4). Supplementation of HeLa NE with K562 NE increased the level of pyneo transcription compared with reactions containing the same total amount of HeLa NE alone (see lanes 1 and 3; and lanes 2 and 4). Interestingly, in a similar in vitro study by Bazett-Jones et al., supplementation of HeLa whole cell extract with K562 NE did not enhance transcription unless the K562 NE was derived from hemin-induced cells. The latter investigators also found it necessary to preincubate K562 NE and template before addition of HeLa NE to achieve stimulated transcription by K562 NE. In contrast, we find that NE from uninduced K562 cells will stimulate transcription and that K562 NE-supplemented reactions consistently yield a higher level of transcription than do reactions containing HeLa NE alone, regardless of whether the template and K562 NE are preincubated.

Because transcription levels were observed to increase in proportion to the amount of supplementary K562 NE present in a reaction mixture (Fig 1, lanes 1 and 2), we next performed reactions in which K562 NE was used as the sole source of transcriptional components. Figure 2 shows the results of these experiments. Transcription in K562 NE
pRSVneo (upper panel) and plasmid pMLP (lower panel) in HeLa (lanes 1 and 2) and in K562 (lanes 3 and 4) NE. Under the reaction conditions used, transcription of pRSVneo was stronger than pMLP, but, more importantly, both of the viral promoter-containing plasmids were transcribed to an equal extent in K562 and HeLa NE. These experiments were repeated with several different preparations and concentrations of K562 and HeLa NE, and with HeLa NE from a commercial source with identical results (data not shown). We conclude, therefore, that K562 and HeLa NE used in these studies possess equivalent transcriptional potency. These results show that the substantially greater transcription of pRSVneo (Figs 1 and 2, above) that we observe in the erythroid K562 NE as compared with the nonerythroid HeLa NE is related to specific recognition of the γ-globin promoter by a component(s) in the K562 NE, and is not due to differences in overall transcriptional efficiency of the two extracts.

Human globin gene transcription from a cosmid construct. We also used a cosmid construct, µLCRγβδβγ

alone is considerably greater than at the same concentration of HeLa NE alone (Fig. 2, lanes 1 and 3; and lanes 2 and 4). These results suggest the presence of γ-globin promoter-specific transcriptional factor(s) in the K562 NE that is either absent, in low abundance, or present in an inactive form in the nonerythroid HeLa NE.

An alternative explanation for this result is that K562 NE has greater overall transcriptional potency than HeLa NE. To eliminate this possibility, we performed reactions in which nonglobin viral promoter-containing plasmids were transcribed in equal amounts of K562 and HeLa NE. We used pRSVneo, containing the Rous sarcoma virus promoter, and pMLP, containing the adenovirus 2 major late promoter. Initially, our attempts to cotranscribe the nonglobin and the γRSVneo templates in the same reaction mixture resulted in low levels of transcription from all templates, giving gel autoradiograph patterns that were not interpretable. We therefore placed the viral promoter constructs in separate reaction mixtures and compared the level of transcription from these templates in K562 and HeLa NE. Figure 3 shows the results of primer extension of RNA synthesized in transcription reactions using plasmid template

HeLa K562

1 2 3 4

Fig 3. Primer extension analysis of nonglobin template transcription in K562 and HeLa NE. Templates for in vitro transcription are plasmids pRSVneo (upper panel) and pMLP (lower panel) (see Materials and Methods). Transcription and primer extension reactions are as described in Fig 1. (Upper panel) Primer is complementary to neo sequences in pRSVneo. Transcription reactions contain 100 µg HeLa NE (lane 1); 170 µg HeLa NE (lane 2); 100 µg K562 NE (lane 3); 170 µg K562 NE (lane 4). (Lower panel) Primer is complementary to the adenovirus 5' untranslated sequences in plasmid pMLP. Lanes 1 through 4 are as in the upper panel. Bands observed are the same sizes (36 to 40 bases) as obtained for pMLP transcribed in vivo (M. Reach, personal communication, 1991).
(see Materials and Methods), as template in an attempt to develop an in vitro transcription assay that could be used to study human fetal-to-adult globin gene switching. With the \( \muLCR^{ay\beta\beta} \) cosmid as template, we found that synthesis of both \( \gamma \)- and \( \beta \)-globin transcripts is considerably stronger in K562 NE (Fig 4, lanes 1 and 2) than in HeLa NE (lanes 3 and 4). We interpret this finding as additional evidence for the globin promoter-specific enhancement of transcription by factors present in K562 NE.

The use of the cosmid template also permits us to assess the relative transcriptional activities of the \( \gamma \)- and \( \beta \)-globin promoters in the fetal environment, because K562 cells express the endogenous fetal (\( \gamma \)-), but not adult (\( \beta \)-) globin genes. With K562 NE, the \( \gamma \)-primed reaction yields the expected band at approximately 75 bp (lane 2, open arrow), indicating specific transcription initiation for \( \gamma \)-globin mRNA. Without template, a weak signal, presumably representing endogenous \( \gamma \)-globin mRNA in the K562 NE, is detected on overexposed autoradiograms (data not shown). In the primer extension reaction with the \( \beta \)-globin–specific primer, a weak signal at approximately 98 bp (lane 1, closed arrow) indicates low-level, but correctly initiated, synthesis from the cosmid \( \beta \)-globin promoter. The corresponding reaction without template indicates that no endogenous \( \beta \) transcript is present in the K562 extract (not shown).

These results indicate that both \( \gamma \) - and \( \beta \)-globin promoters of the cosmid template are active in K562 NE, but that greater levels of transcription occur from the \( \gamma \)-promoter (Fig 4, lane 2, open arrow) than the \( \beta \)-promoter (lane 1, closed arrow). This is consistent with the predominant embryonic and fetal globin expression characteristic of intact K562 cells.

The effect of competitor oligonucleotides on in vitro transcription from the \( \gamma \)-globin promoter. In vitro assay system was also used to address the role of various trans-acting factors in \( \gamma \)-globin gene transcriptional regulation; an issue that has not been resolved to date. In our in vitro transcription system, the addition of a double-stranded oligonucleotide containing the recognition sequence for a transcriptional activator will result in decreased transcription, whereas the addition of an oligomer with the binding site for a repressor will result in increased transcription, due to competition between template and oligomer binding sites for specific binding proteins present in the nuclear extract. Specifically, we were interested in determining the effect of competing the ubiquitous octamer-binding protein, Oct-1, on \( \gamma \)-globin transcription. It has been proposed that Oct-1 may function as a repressor of \( \gamma \)-globin transcription, because a naturally occurring mutation of an octamer sequence at the \( -175 \) position in the \( \gamma \)-globin promoter results in the gene’s increased expression in adult erythroid cells (hereditary persistence of fetal hemoglobin [HPFH]). However, this hypothesis has been weakened by a mutational analysis of the \( -175 \) region, that suggested that the increased expression was mediated by the erythroid transcription factor GATA-1, which has an overlapping binding site in this region. An in vitro transcription assay using Oct-1 competitor oligonucleotides would provide a different approach to attempt to answer this unresolved question.

Figure 5 shows the products obtained by primer extension of transcripts synthesized in vitro from \( \gamma neo \) in the presence (lanes 3 and 7) or absence (lanes 1 and 5) of a double-stranded oligomer containing the octamer consensus binding sequence (ATGGCAAAT). This oligomer has previously been shown to bind Oct protein in gel mobility shift assays. Reaction mixtures containing the octamer oligonucleotide (lanes 3 and 7) yield increased transcription levels as compared with those that do not include the oligomer (lanes 1 and 5). In 28 different experiments, 22 showed the same or an even greater increase in transcription as shown in Fig 5. The results support the hypothesis that octamer protein functions as a repressor of \( \gamma \)-globin gene transcription.

Control reactions using a double-stranded oligomer containing a 3-base mutation within the octamer consensus binding sequence (Fig 5, lanes 4 and 8) show that addition of the mutant octamer binding site results in transcription levels equivalent to those of the baseline reaction (lanes 1 and 5). The same mutant octamer sequence has been shown to be incapable of binding the Oct protein in gel shift experiments. As a positive control, we performed competition...
show the occurrence of preferential transcription directed from globin promoters in K562 as compared with HeLa NE. With the cosmid template, γ-globin gene transcription is much greater than β-globin gene transcription, consistent with the embryonic/fetal pattern of expression of the endogenous globin genes in K562 cells, and with previous findings by our laboratory33,34 and others12,35 of higher ratios of γ-to β-promoter–initiated transcripts in K562 cells transfected with human β- and γ-promoter/neo constructs.

Bazett-Jones et al18 first showed that hemin-induced K562 NE (but not uninduced K562 NE) can enhance globin promoter-specific transcription when they used K562 NE to supplement HeLa NE in an in vitro transcription assay. Unexpectedly, all of the globin promoters that they tested (ε, γ, and β) were transcribed at approximately equal levels, even though K562 cells do not express the endogenous β-globin gene. It was proposed that this difference might be due to the absence of the influence of “epigenetic factors” such as chromatin structure or specific chromatin-bound nonhistone proteins in in vitro experiments.18 Later, Wada and Noguchi19 were able to show that K562 NE alone (both induced and uninduced), without supplementation with HeLa NE, could direct globin promoter-specific enhanced transcription.19 In contrast to the earlier work by Bazett-Jones et al,18 they documented differential transcription from the human ε-, γ-, and β-globin promoters, in the order of $\epsilon > \gamma \approx \beta = 0$. It was hypothesized that this observed difference between their results and those of Bazett-Jones et al could be explained by the use of HeLa NE in the earlier experiments. More recently, Fischer and Nowock20 have described the use of NE from a number of erythroid cell lines, including K562 cells, in in vitro transcription assays. They found a slightly higher level of γ-globin promoter-specific transcriptional activity in the NE of K562, HEL (human fetal erythroid), and B8/3 (mouse adult erythroid) as compared with HeLa NE. The observation of accurately initiated γ-globin transcripts using NE of the B8/3 cell line, which expresses only the β-globin gene, again showed a loss of developmental stage-specificity in the in vitro assay, similar to the findings of Bazett-Jones et al and conflicting with those of Wada and Noguchi. Furthermore, the demonstration that HeLa NE alone can produce accurately initiated γ-globin transcripts suggests that cell-free in vitro transcription assays may not completely reproduce the tissue-specific and developmental-stage-specific transcriptional regulation of genes seen in vivo, as proposed by Bazett-Jones et al.18

In the present study, we document significantly enhanced globin promoter-specific in vitro transcription using uninduced K562 NE as compared with parallel experiments using HeLa NE, using both plasmid pγneo and the μLCR4γψβbβ cosmid as template. We also show enhanced transcription of the γ-globin gene as compared with the β-globin gene using the cosmid template. Although it is likely that this finding is related to the presence of γ-promoter–specific transcription factors in K562 NE, it is also possible that the γ- and β-globin promoters have inherently different strengths, because we found slightly higher levels of γ- than β-globin–specific transcription using HeLa NE.

experiments using a double-stranded oligomer containing the Sp1 consensus binding sequence. In these reactions there was markedly decreased transcription compared with baseline (Fig 5, lanes 2 and 6 compared with lanes 1 and 5). The results for Sp1 oligomer effect on transcription are as expected, consistent with the function of Sp1 (or CAC box factor) as an activator of transcription for several eukaryotic genes, including the γ-globin gene.10,26,29-32 Sp1 has also been shown to be involved in the activation of the human γ-globin promoter in transient transfection assays.6,16 in a transgenic mouse model,10 and, recently, in an in vitro transcription competition assay.20 In other experiments using GATA-1 and CCAAT oligomers, no consistent effects on transcription were seen.

DISCUSSION

Using a cell-free in vitro transcription assay system, we have shown that NE from the human erythroid cell line K562 gives rise to enhanced transcription from globin gene promoters as compared with NE from the nonerythroid (epithelial) HeLa cell line. The observed differential transcription is most likely due to the presence of globin promoter–specific transcription factors in K562 NE, because control reactions using nonerythroid viral promoters show synthesis of equivalent amounts of transcripts in both K562 and HeLa NE. Using a cosmid construct template containing the human γ- and β-globin genes and the LCR, we similarly
(Fig 4, lanes 3 and 4). Similar findings with \( \gamma \) and \( \beta \)-globin promoter-containing constructs transfected into HeLa cells have been reported by others.\(^{12}\)

As opposed to the results of Wada and Noguchi,\(^{19}\) but in concordance with those of with Bazett-Jones et al\(^{18}\) and Fischer and Nowock,\(^{20}\) we find accurate initiation of transcription of the \( \beta \)-globin gene of the cosmid construct. There are a number of possible explanations for this observation. It is possible that such transcription is related to the presence of linked upstream LCR sequences, in concordance with the consensus view of the LCR as a pan-globin locus activator. It is also possible that the presence of linked 5'\( \gamma \)-globin promoter sequences may enhance transcription of the \( \beta \)-globin gene. With respect to this suggestion, Lin et al\(^{19}\) detected \( \beta \)-globin gene expression from intact K562 cells transfected with constructs carrying the normal \( \beta \)-globin gene plus 5' flanking regions of the \( \gamma \)-globin gene, and attributed correct initiation of \( \beta \)-globin RNA synthesis to the presence of the linked upstream \( \gamma \) sequences. Finally, it may simply be that in vitro assays using the cosmid template, although a closer approximation of the nuclear environment, may still be lacking such epigenetic factors as are necessary to completely reproduce the appropriate developmental regulation of globin genes seen in vivo.

Fischer and Nowock\(^{20}\) first introduced the use of competitor oligonucleotides in in vitro transcription assays using erythroid nuclear extract. They used the oligomer competition assay to show that the ubiquitous transcription factor Sp1 functions to enhance transcription from the \( \gamma \)-globin gene promoter, probably by interacting with the promoter CACCC box.\(^{20}\) We were able to reproduce these findings in our assay using the pyneo template. Furthermore, we show that addition of a double-stranded oligonucleotide bearing the consensus recognition sequence for the octamer (Oct-1 and -2) DNA-binding proteins results in an increased level of transcription above baseline. Control reactions using a mutant Oct oligonucleotide showed no effect on pyneo transcription. These results indicate that a factor with octamer motif-binding activity can repress transcription from the \( \gamma \)-globin gene promoter. Our findings conflict with those of Martin et al,\(^{2}\) who reported that a mutational analysis of the overlapping \( \gamma \)-globin A and B binding sites at the -170 to -190 region of the \( \gamma \)-globin promoter showed that mutations that abolish the binding of Oct protein but not \( \gamma \)-globin A to the region do not affect the level of transcription. The difference between our results could be explained by a recent report that an octamer protein interacts with a second site further upstream (−260 to −200) on the \( \gamma \)-globin promoter.\(^{16}\) It is, therefore, possible that the octamer-mediated repression of \( \gamma \)-globin transcription that we have observed may be via this upstream sequence within the \( \gamma \)-globin promoter, rather than at (or in conjunction with) the −175 location. It is also possible that octamer protein may exert its effect indirectly by interacting with other proteins that bind to the \( \gamma \)-globin promoter.

Curiously, we were unable to show an effect on pyneo transcription by the addition of a competitor oligonucleotide containing the \( \gamma \)-globin A consensus recognition sequence, even though \( \gamma \)-globin A has been shown to function as a transcriptional activator by cotransfection assays in heterologous cells.\(^{36}\) It is possible that our system is not sufficiently regulated to the extent of exhibiting all in vivo phenomena. However, it should be noted that there is no direct evidence for GATA-1 activation of an intact globin promoter by in vivo trans-activation studies,\(^{36}\) so a negative finding with the GATA-1 oligomer does not necessarily reflect a failure of our in vitro system.

The interplay of nuclear proteins that regulate human \( \gamma \)-globin transcription remains poorly understood. The methodology presented in this report may allow these interactions to be investigated in greater detail. The \( \mu \)LCRA\( \gamma \beta \beta \) cosmidi construct, in particular, with the \( \gamma \) and \( \beta \)-globin genes arranged with respect to each other and to the LCR approximately as they are in vivo, offers a unique opportunity, in conjunction with oligonucleotide competition experiments, for the in vitro investigation of trans-acting factors involved in fetal-to-adult human hemoglobin switching. We are hopeful that our findings with this template could represent a starting point for further exploration along these lines.

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