CAAT/Enhancer-binding Proteins Are Involved in β-Globin Gene Expression and Are Differentially Expressed in Murine Erythroleukemia and K562 Cells*

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Acting in cis with the β-globin locus control region, the CAAT box of the β-globin gene promoter stimulates transcription 10-fold in murine erythroleukemia (MEL) cells but is without effect in K562 cells. Our previous studies suggested that four proteins from MEL cells that bind to this CAAT box region (CP1, GATA-1, and two factors that were denoted DSFr and DSF1) DSFr is involved in the up-regulation of transcription. In the present report, the DSFr protein in MEL cells was identified as C/EBPγ through expression cloning and antibody studies. C/EBPγ DNA binding activity could not be detected in K562 cells. However, K562 cells, but not MEL cells, were found to express LIP, which is a truncated form of C/EBPβ and is an inhibitor of transcription. Thus, the differential expression of C/EBP members could account for the ability of the β-globin CAAT box to stimulate transcription in MEL cells, but not function in K562 cells. Juxtaposing a specific C/EBP binding sequence next to the β-globin promoter, in constructs in which the CAAT box had been rendered inactive by mutation or deletion, restored full promoter activity in MEL cells only if CP1 still bound to the promoter. In conjunction with previous mutation analyses, these results suggest that C/EBPγ may collaborate with CP1 to enhance transcription through the β-globin CAAT box.

The human β-globin locus (Fig. 1) contains five active genes that are expressed specifically in red blood cells at different times during development (reviewed in Ref. 1). ε-Globin is only expressed early in embryogenesis, the two γ-globin genes are transcribed at high levels during fetal life, while the δ- (a minor contributor) and β-globin genes are expressed in late fetal life and throughout adult life. To express at high levels, all of these genes rely on the activity of the LCR1 that is situated upstream of the ε-globin gene (see Fig. 1). The LCR is a powerful, erythroid-specific enhancer that has the additional property of being able to confer position-independent, copy number-dependent expression onto globin transgenes under its control (reviewed in Ref. 2).

Transgenic mouse studies of the β- and γ-globin genes, alone or together in cis with the LCR (3–6), as well as studies of the whole human β-globin locus (7, 8), have shown that the globin genes compete for activation by the LCR during development. Investigations have also indicated that the LCR can only activate one gene at a time and that the LCR interacts directly with the gene it is enhancing through a looping mechanism (3, 6, 9). Thus, it is believed that the developmental switching of globin gene expression is regulated, at least in part, through a competition between the genes for interactions with the LCR. In this model, regulatory sequences surrounding each gene would interact with the LCR and, therefore, would play a direct role in determining the affinity each gene has for the LCR during the different stages of development.

Using MEL cells as a model system, Antoniou and Grosveld (10) demonstrated that only a minimal β-globin gene promoter, containing the TATA box, the CAAT box, and the proximal CACC box sequences (Fig. 1), is necessary for the LCR to enhance high levels of transcription onto a H-2Kβ reporter gene. In conjunction with the TATA box, the CAAT, and the proximal CACC motifs can each stimulate activity 10-fold higher than the TATA box alone in MEL cells. Thus, both the CAAT and proximal CACC elements and the cognate transcription factors that bind to these sequences play an important role in regulating the β-globin promoter in the presence of the LCR and, thus, may be involved in LCR interactions.

Recent work by other groups has implicated an erythroid-specific kruppel-like factor in the involvement of the β-globin proximal CACC box in LCR activated transcription (11, 12). Mice in which the erythroid-specific kruppel-like factor gene has been crippled though homologous recombination died at the fetal stage of development due to a severe anemia. This may be caused by the specific absence of expression of the mouse β-globin gene (13, 14).

Our studies on the β-globin CAAT box have shown that four distinct factors in MEL cells can bind to this region; the erythroid-specific protein GATA-1, the ubiquitous CAAT-binding protein CP1 (also called NF-Y), and two DNA binding activities that were denoted DSF1 and DSFr (15). DSF1 DNA binding activity is only detectable in MEL cells that are induced to terminally differentiate, while DSFr activity is present in both uninduced and induced cells. A mutation analysis that was done suggested that DSFr is involved in regulating the activity of the β-globin CAAT box in MEL cells. However, in these same studies it was also found that the CAAT box, in cis with the LCR, was completely unable to increase transcription from the β-globin promoter in K562 cells (15). Yet, it appeared that K562 cells did contain DSFr DNA binding activity. These results suggested that there might be a difference in the way the LCR functions in MEL versus K562 cells and that there are differences in the CAAT box-binding factors that were not detected in the previous studies. Therefore, we sought to identify the DSFr and DSF1 proteins to be able to determine if there...
exist important differences between the proteins in MEL cells versus K562 cells.

The C/EBP family of transcription factors belong to the basic-ZIP class of DNA-binding proteins (Refs. 16 and 17, and references therein). C/EBP members contain a domain in their C-terminal half that is rich in basic amino acids. This basic domain is followed by a leucine zipper motif (4 to 5 repeats of leucine at every seventh amino acid) through which the C/EBP members form homodimers or heterodimerize with one another. Dimerization is required for the proteins to interact with DNA through the basic domains.

At least six distinct members of the C/EBP family in mammals have been identified: C/EBPα, C/EBPβ (also known as NFIL6 and LAP), NFIL6β, C/EBPγ (also called IgEβ), C/EBPδ, and CHOP (16–24). With the exception of CHOP, which does not appear to bind DNA (22), all C/EBP members have a nearly identical DNA-binding specificity. Although the DNA sequence consensus recognized by these proteins is highly redundant, a CAAT-like sequence is frequently observed. In the present report we have identified DSF1 and DSFr as C/EBPβ and C/EBPγ, respectively, and we have found that these DNA binding activities are differentially expressed in MEL and K562 cells. Moreover, our studies suggest that C/EBPβ may act in collaboration with CP1 to up-regulate LCR enhanced β-globin gene expression through the β-globin CAAT box in MEL cells.

### MATERIALS AND METHODS

Library Screening—Custom cDNA libraries, representing dimethyl sulfoxide-induced MEL cells, in λgt11 (random primed) and λgt10 (mixed random and oligo(dT) primed) vectors were purchased from Clontech. For screening λgt11 expression libraries, approximately 50,000 plaque-forming units were grown on each 10-inch square plate and these were screened by the Southwestern method (25). Filters were probed with the synthetic oligonucleotides GAGCCCTAGGGTGGC-CATCTACTCCGAC and GCTCTGGAGGATAGATTGGCCAACCCTAGG, which were end labeled with [γ-32P]ATP, annealed to form GAGC and GCTC 5' overhangs, and then ligated to form concatamers. Gel Mobility Shift Assays—GMS assays and nuclear extract preparation were done as described previously (15). Extracts were used at a protein concentration of 0.8 mg/ml in the final reaction mixtures unless indicated otherwise. Antisera were diluted 3-fold in phosphate-buffered saline and then extracts were mixed with equal amounts of diluted antisera and incubated on ice for 30 min. Antibodies to C/EBPα were kindly provided by Kathryn Calame (26). Antibodies to C/EBPβ and C/EBPγ were purchased from Santa Cruz Biotechnology Inc. Antibodies to GATA-1 were as described (27). The β-CAAT oligonucleotide used and mutants 1, 3, 5, and 7 of these sequence have been described previously (15). The 5' oligonucleotide was (both strands are given 5' to 3') ATTTGATTATGGCAACCTTGTATGACAATGTGATAACAGTGGAA-CATAACAAA.

Synthesis of DNA Constructs—Plasmid constructs containing the β-globin promoter from position −87, which is just upstream of the CAAT box (Fig. 1), to +32 linked to a H-2K reporter gene and cloned into the β-globin microcosmic vector, which contains the β-globin LCR, have been described previously (15). A promoter containing a deleted CAAT box was synthesized by the polymerase chain reaction using a 5' primer with the sequence 5'-CCCGGGTATCCTACTCCAGGACAGGG-3'. The underlined sequence represents a BamHI site that was added for cloning purposes and the T following this represents the start of the sequence of the β-globin promoter beginning two nucleotides 3' to the CAAT sequence. The DNA product was linked to the H-2K reporter and cloned into the microcosmic vector as described (15). To clone the CPS sequences 5' to the various β-globin promoters, the CPS oligonucleotides (given above) were re-synthesized to contain a 5' CG overhang on the first strand and a 5' GATC overhang on the second strand. The oligonucleotides were annealed and then cloned juxtaposed 5' to the promoters using BamHI and Clal restriction sites that are unique when the promoter fragments are cloned into BlueScript vector before they are transferred to the microcosmic vector (15). The final products were sequenced to ensure only one copy had inserted and that it was in the correct orientation. The rat LIP expression vector was kindly provided by Ueli Shibli (21).

Western Blots—Fifty μg of proteins from nuclear extracts were separated on 13% SDS-polyacrylamide gels, the proteins were transferred electrophoretically to nitrocellulose membranes and the membranes were probed with a 1:2000 dilution of C/EBPβ antibodies using the ECL system of Amersham Life Sciences.

### RESULTS

DSF1 and DSFr in MEL Cells Are C/EBPβ and C/EBPγ, respectively—To clone the corresponding cDNAs for the DSF proteins, a λgt11 expression library was screened with a DNA probe representing the CAAT box of the β-globin promoter. One positive clone was obtained from 0.8 × 10⁶ recombinants screened. This clone contained part of the open reading frame and 3' sequences of the C/EBPγ cDNA that was previously cloned from mouse and rat cells (18–20). A λgt10 cDNA library was screened to isolate the complete coding sequences for C/EBPγ. Interestingly, the 5' end of the longest cDNA isolated extended to the G 39 base pairs upstream from the C that was suggested to be the site of transcription initiation for the C/EBPγ gene (Ref. 18, data not shown). This result agreed with studies on rat C/EBPγ, in which the 5' end of the longest cDNA isolated is highly homologous to the mouse gene and extends 5' to just 2 nucleotides before our longest clone (20). Thus, either the transcription start site for the mouse C/EBPγ gene was incorrectly mapped or multiple start sites are used.

To determine if DSF1 and/or DSFr represent C/EBP members, MEL nuclear extracts were incubated with antibodies against various C/EBP proteins before performing GMS assays with the β-globin CAAT box oligonucleotide as the labeled DNA probe. To identify the C1, DSF1, GATA-1, and DSFr complex on the autoradiograms, competition assays were performed (Fig. 2a) with various mutations of the CAAT box that were previously described (15). These can be summarized as follows: CP1, GATA-1, DSF1 and DSFr bind to the wild type β-CAAT sequence; only GATA-1 recognizes mutant 1; mutant 3 is recognized by DSFr and DSF1, and, to a greatly reduced degree, by CP1; while only CP1 binds to the mutant 7 sequence. Each oligonucleotide will only compete binding of those proteins that bind to it.

With uninduced MEL extracts (Fig. 2b, lanes 1–6) the C/EBPγ antisera inhibited formation of the DSFγ protein-DNA complex and caused a supershift to form, but it did not affect either GATA-1 or CP1 (lane 3). None of the bands were affected by antisera against C/EBPβ or C/EBPδ, or by preimmune serum (lanes 4, 5, and 6, respectively), while GATA-1 antibodies specifically inhibited formation of the GATA-1 complex (lane 2). With induced MEL extracts (Fig. 2b, lanes 7–12) formation of the DSFγ complex was again only inhibited by the antibodies against C/EBPγ. The C/EBPγ antisera also partially inhibited formation of the DSF1 complex (lane 9). In addition, the DSF1 band was completely inhibited by the C/EBPβ antisera (lane 10). However, the DSF1 signal was not affected by GATA-1 antisera, C/EBPδ antisera, or preimmune serum.
Fig. 2. DSF1 and DSFr are C/EBP family members. In a, GMS assays were performed with uninduced (lane 1) or induced (all other lanes) MEL extracts with the \( \beta \)-globin CAAT sequence as the DNA probe, in the absence of competitor (−) or in the presence of a 100-fold molar excess of the competitor oligonucleotide shown at the top of each lane. Mutant 1 binds GATA-1 only, mutant 3 binds DSF1 and DSFr (CP1 very weakly), mutant 7 binds CP1 only (15). The CPS sequence binds to DSF1 and DSFr specifically (see text). In b, uninduced (lanes 1–6) or induced (lanes 7–12) MEL extracts were preincubated with the antiseraum shown at the top of each lane before performing the GMS assays. −, no antiseraum.

(lanes 8, 11, and 12, respectively), which demonstrated that the effects were specific.

To extend these results, we determined if the DSF1 and/or DSFr factors could recognize DNA sequences known to interact with C/EBP members. A GMS analysis using a sequence from the promoter of the CPS gene, which interacts strongly with C/EBP proteins (28), is shown in Fig. 3. Two bands were detected in induced MEL extracts (Fig. 3a, lane 2) that showed exactly the same competition profile as DSFr and DSF1 detected with the \( \beta \)-CAAT probe (compare lanes 2 to 7 in Figs. 2a and 3a). The faster migrating band was virtually completely inhibited by the anti-C/EBP\( \gamma \) serum, but this band was not affected by any of the other sera tested, while the upper band was partially affected by the anti-C/EBP\( \gamma \) serum and was completely inhibited by the anti-C/EBP\( \beta \) serum (Fig. 3b, lanes 6–10). The apparent slight reactivity of the upper band to anti-C/EBP\( \beta \) and the slight decrease in the lower band in response to anti-C/EBP\( \beta \) and anti-C/EBP\( \gamma \) was not reproducible. Thus, the two factors in induced MEL extracts that bound to the CPS oligonucleotide gave the same DNA-binding specificity and the same antibody reactivity as DSFr and DSF1 that bind to the \( \beta \)-globin CAAT sequence.

Surprisingly, the CPS oligonucleotide also formed two DNA-protein complexes in GMS assays with uninduced MEL extracts (Fig. 3a, lane 1). However, the slower migrating band seen with uninduced extracts migrated at a slightly higher position than the slower migrating band seen with induced extracts (compare lanes 1 and 2 in Fig. 3a), and antibody studies indicated that the upper band obtained with uninduced extracts was likely due to two molecules of DSFr binding to a single molecule of the CPS DNA probe. For example, both of the DNA-protein complexes detected with uninduced extracts were almost completely blocked by the anti-C/EBP\( \gamma \) serum, whereas neither was affected by the other sera tested, including the C/EBP\( \beta \) antiseraum (Fig. 3b, lanes 1–5 and compare lanes 3 and 4 to lanes 8 and 9). This was in contrast to the upper band obtained with induced extracts, which was completely reactive to C/EBP\( \beta \) antiseraum.

In summary, both DSFr and DSF1 DNA binding activities can be detected with C/EBP-binding sites, as well as with the \( \beta \)-globin CAAT sequence, in GMS assays. DSFr is specifically reactive to C/EBP\( \gamma \) antibodies. DSF1 is partly reactive to C/EBP\( \gamma \) antibodies and it is completely reactive to C/EBP\( \beta \)-specific antibodies. These data suggest that DSFr is C/EBP\( \gamma \) in MEL cells, while DSF1 is likely C/EBP\( \beta \). If DSF1 is C/EBP\( \beta \), it must be present in part as a homodimer and in part as a heterodimer with the C/EBP\( \gamma \) (DSFr) protein. This would explain why it is partly reactive to the C/EBP\( \gamma \) antiseraum. This interpretation is exactly the same conclusion drawn by Cooper et al. (26) for mouse B-cells. Both early and mature B-cells contain active C/EBP\( \gamma \), but only more mature cells contain detectable C/EBP\( \beta \) DNA binding activity and a large proportion of the C/EBP\( \beta \) protein is present as a heterodimer with C/EBP\( \gamma \), as appears to be the case here for MEL cells.

K562 Cells Contain LIP, a Truncated Form of C/EBP\( \beta \) That Is an Inhibitor of Transcription—K562 cells were also studied using antisera and GMS assays as outlined above for MEL extracts. In panel a of Fig. 4, the identity of the various radio-labeled bands obtained in GMS assays of K562 extracts using the \( \beta \)-CAAT oligonucleotide as the probe was established by competition assays, while the effect of preincubation with the various antibodies is shown in panel b of this figure. Previously (15), we found that GATA-1, CP1, and a band migrating near the position of and showing the same competition pattern as DSFr and DSF1 were observed upon treatment with this antiserum (Fig. 4b, lane 3). With the CPS oligonucleotide as probe, only one major band migrating at the position of DSFr was not at all reactive to the C/EBP\( \gamma \) antiseraum and treatment with this serum did not cause any super shift to form (lane 3 in Fig. 4b). This indicated that none of the complexes seen with K562 extracts is related to C/EBP\( \gamma \) (DSFr). On the other hand, the protein-DNA complex seen at the DSFr position was strongly inhibited by the C/EBP\( \beta \) antiseraum and supershifted bands were observed upon treatment with this antiseraum (Fig. 4b, lane 4). With the CPS oligonucleotide as probe, only one major labeled protein-DNA complex was obtained in GMS assays of K562 extracts (Fig. 4c; minor bands observed were not reproducible). This complex gave the competition profile expected for a C/EBP protein (Fig. 4, compare results in panel c to those in panel a) and again, this DNA-protein complex was reactive to the C/EBP\( \beta \) antibodies specifically (Fig. 4d). Thus, the DSF related complex observed with K562 extracts is related to C/EBP\( \beta \) (DSF1), not to C/EBP\( \gamma \) (DSFr).

The C/EBP\( \beta \) mRNA contains three in-frame methionines that can be used as protein initiation codons in vivo (21). The first two ATGs are near the 5’ end of the message and code for proteins in the 35–40-kDa range. The third ATG is near the center of the open reading frame and codes for a protein of only 16 kDa. This protein, called LIP, migrates upon SDS-polyacryl-
amide gel electrophoresis near 20 kDa. Since in the GMS assays the C/EBP-related DNA binding activity in K562 extracts co-migrates with C/EBPγ (DSFr) from MEL cells, and C/EBPγ itself is a 16.3-kDa protein (18), it was suspected that the K562 DNA binding activity might represent LIP. In Western blots probed with C/EBPβ antibodies (Fig. 5a), a prominent band migrating near 20 kDa was seen with K562 extracts, whereas no protein at this position was detected with MEL extracts. Moreover, in a K562 clone that was stably transfected with an expression vector for the LIP protein of rat, the intensity of this 20-kDa protein detected in Western blots with the C/EBPβ antibodies was increased substantially (Fig. 5b). In GMS assays of extracts prepared from this clone, an intense DNA-protein complex, migrating near the position of the DSF-related band seen with normal K562 extracts, was obtained with both the β-CAAT and CPS sequences as probes (Fig. 5c). Note that the major band seen with the LIP expressing clone migrated slightly above the position of the DSF-related activity seen with normal K562 cells. This likely represents a difference in migration between the transfected rat LIP protein and the endogenous human K562 protein. Taken together, these data conclusively show that the DSF-related DNA binding activity in K562 extracts represents the LIP protein. LIP is present in K562 cells but not in MEL cells.

In the Western blot analyses, the C/EBPβ antiserum also detected major protein bands in the 35–40-kDa range with both K562 and MEL extracts and these were detected equally well with uninduced and induced extracts from either cell type (Fig. 5a). It is expected that these represent the larger C/EBPβ proteins (see above). Yet, C/EBPβ (DSF1) DNA binding activity can only be detected with induced extracts of MEL cells. It cannot be detected with uninduced MEL extracts, nor with any K562 extracts (see Figs. 2 and 4 for example). Thus, it was thought that only induced MEL cell extracts would contain full-length C/EBPβ proteins. However, the Western blot analyses suggest that both K562 and MEL cells, whether they are induced or not, contain the full-length proteins. One possibility is that the C/EBP proteins are not active for DNA binding. For example, it has been shown that the DNA binding activity of C/EBP proteins can be modified by phosphorylation (29). In this respect, a minor band migrating near 35 kDa is detected with induced MEL extracts by the C/EBPβ antibodies in the Western blot analysis, but this band is not detected with uninduced MEL or any K562 extracts (arrow in Fig. 5a). Thus, it may be that it is a specific form of the C/EBPβ protein that is active for DNA binding in induced MEL cells. Further studies will be required to address this possibility.

An Oligonucleotide That Binds Specifically to the C/EBP Factors Can Reconstitute the β-Globin CAAT Box, but Only in Conjunction with CP1—Our previous results (15) had sug-
Fig. 5. K562 cells contain LIP. In a, equivalent amounts of protein extract from K562 or MEL cells, were separated by SDS-polyacrylamide gel electrophoresis and then analyzed by Western blot using antibodies against C/EBPβ. The position of protein molecular weight standards run in parallel are indicated on the left-hand side, and the source of the extracts are indicated at the top of the lanes. Note that LIP is the 20-kDa protein band that is only seen in the K562 lanes, while the major bands near 40 kDa seen in all lanes are expected to be the larger forms of C/EBPβ. The arrow to the left indicates the position of a band that is only seen with induced MEL extracts. −, uninduced extracts; +, induced extracts. In b, extracts from normal K562 cells (lane 1), or from a K562 cell clone transfected with a rat LIP expression vector (lanes 2–4) were analyzed by Western blot as in a. In c, GMS assays were performed using extracts from normal K562 cells (lanes 1 and 3) or the LIP expressing clone of K562 cells (lanes 2 and 4) with either the β-CAT oligonucleotide (lanes 1 and 2) or the CPS oligonucleotide as the DNA probe. Extracts were used at a final protein concentration of 0.2 mg/ml to emphasize the difference in DNA binding activity.

suggested that C/EBPβ is involved in LCR enhanced activation of transcription through the β-globin CAAT box in MEL cells. We, therefore predicted that a DNA sequence that binds specifically to C/EBP proteins in MEL cells, such as the CPS oligonucleotide (Fig. 3), may be able to reconstitute transcriptional activity to a β-globin promoter in which the CAAT box had been inactivated. Previously, we had derived several multiple point mutations of the β-globin CAAT sequences (15). These were investigated using constructs that contained the LCR in cis to the β-globin promoter (from −87 to +32, which includes just the TATA box and CAAT box motifs (Fig. 1)) linked to a H-2K reporter gene. Two of the CAAT box mutations studied, mutant 5, which no longer binds to any proteins, and mutant 7, which only retains CP1 binding, had a 10-fold reduced activity in LCR enhanced transcription in MEL cells, suggesting that in these two cases the β-globin CAAT box is not at all functional (Ref. 15; a summary of these two mutations is shown in Fig. 7). In addition, we have derived an additional construct in which the CAAT box and further upstream sequences were deleted from the β-globin promoter (−71 to +32). This deleted promoter, denoted deleted CAAT, also had a 10-fold reduced activity in MEL cells (see below). We tested whether juxtaposing the CPS oligonucleotide 5′ of the mutant 5, mutant 7, or deleted CAAT promoters could restore transcription in MEL cells to the levels seen with the wild type promoter extending to −87. The promoter fragments (with or without the CPS sequence juxtaposed 5′ to them) were linked to a H-2K reporter gene in the LCR containing microcosmic plasmid. Stably transfected populations of MEL cells were selected and then dimethyl sulfoxide-induced populations were assayed by RNase protection (Fig. 6) for expression of the H-2K reporter mRNA relative to GATA-1 mRNA as an internal control. The RNase protection signals were quantified and corrected for the relative transgene copy number (which was determined by Southern blot analysis) in each population. A summary of the promoter constructs tested, what factors each binds to, as well as the expression results, is given in the top part of Fig. 7. The CPS sequences did not stimulate activity of either the deleted CAAT or the mutant 5 promoter. In contrast, the CPS oligonucleotide did stimulate the mutant 7 promoter approximately 8-fold, such that the promoter was now as active as the wild type promoter. None of the constructs gave any appreciable activity in K562 cells (results not shown).

To ensure that the ability of the CPS sequence to stimulate transcription of the mutant 7 promoter in MEL cells was not because a new protein binding site had been created, polymerase chain reaction was used to synthesize each promoter fragment containing the CPS sequence (CPS-del CAAT, CPS-mutant 5, and CPS-mutant 7) and these were studied by GMS assays with extracts from MEL cells (Fig. 8). The results showed that in addition to CP1, C/EBPβ, and C/EBPγ, the CPS-mutant 7 DNA fragment formed one additional specific DNA-protein complex. This complex migrated above the CP1 position on the gels (marked C/EBP-CP1 in the figure). Other bands observed were not reproducible and/or were nonspecific. For example, the band migrating below the C/EBPγ position was not observed in all experiments and it was nonspecific, as it could be competed to some degree by all competitors tested, especially mutant 5 and mutant 7. Competition assays showed that the band migrating above CP1 represented CP1 and C/EBPγ or β binding together on the CPS-mutant 7 DNA fragment. For example, it was competed by the CPS, wild type β-CAAT, and mutant 7 oligonucleotides, but it was not inhibited by mutant 5 (lanes 3–7 in Fig. 8). In addition, in competing with CPS (competes C/EBP proteins specifically), the band at the position of CP1 increased (lane 5), while in competing with
mutant 7 (competes CP1 specifically) the bands at the positions of C/EBP \( \gamma \) and C/EBP \( \delta \) increased (lane 7). Moreover, when the CPS-mutant 7 fragment was competed with the CPS oligonucleotide, the GMS pattern observed exactly coincided with the pattern obtained for CPS-mutant 5 and CPS-deleted CAAT (compare lane 7 to lanes 1 and 2 in Fig. 8), showing that the only extra protein that binds to CPS-mutant 7, relative to these two other promoter fragments, is CP1.

**DISCUSSION**

In previous studies (15) we showed that whereas the wild type CAAT box of the \( \beta \)-globin promoter can stimulate LCR-enhanced transcription 10-fold in MEL cells, the CAAT box is completely inactive in K562 cells. Mutation and stability analyses indicated that a factor that was then denoted DSFr is involved in the CAAT box function in MEL cells. In the present report, we showed that the C/EBP \( \gamma \) transcription factor represents the DSFr DNA binding activity detected in MEL cells. However, no C/EBP \( \gamma \) DNA binding activity could be detected with extracts from K562 cells, showing that K562 cells do not contain active C/EBP \( \gamma \). We cannot say whether or not K562 cells contain inactive C/EBP \( \gamma \) protein, as the antibodies to this protein did not work well in Western blot analyses. However, K562 cells were found to contain substantially less C/EBP \( \gamma \) mRNA than MEL cells (not shown), suggesting that the regulation might be at the level of transcription. Further studies will be necessary to clarify this point.

The absence of active C/EBP \( \gamma \) in K562 cells might be expected to account for the inability of the \( \beta \)-globin CAAT box to function in cis with the LCR in these cells. However, with clones of K562 cells that were stably co-transfected with a \( \beta \)-globin promoter-LCR reporter plasmid (the promoter from −87 to +32 with an unmutated CAAT box) and a C/EBP \( \gamma \) expression plasmid, we failed to detect any expression of the

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**FIG. 7.** Both CP1 and C/EBP\( \gamma \) are required for wild type levels of expression. In the top part of the figure, the \( \beta \)-globin promoter constructs studied in this report are summarized along with the factors that bind to them. The CAAT box region is outlined, mutations from the wild type \( \beta \)-globin sequence are shown in bold lower case letters, and a BamHI restriction enzyme site (ggatcc) that was used to done in the CPS sequence is shown in smaller lower case letters. The consensus C/EBP-binding site in the CPS sequence is also outlined. To the right of the figure, the average level of expression per transgene copy (±, average variation) of each construct, as measured by RNase protection and Southern blot analysis, is given. Each value is an average from the two different transfected populations shown in Fig. 6, except for the mutant 5 + CPS, mutant 7 + CPS constructs, for which three, three, and four different populations were studied, respectively. (Note that the expression values given here may not appear to correspond to the expression levels in Fig. 6. However, the calculated values have been corrected for copy number since the LCR confers copy number-dependent expression onto transgenes in MEL cells (36). For example, deleted CAAT + CPS would appear to have a much lower level of expression than mutant 5 in Fig. 6. However, the average copy number for the mutant 5 populations was approximately 5-fold higher than for the deleted CAAT + CPS populations. Upon correction, they turn out to have approximately the same level of expression.) In the bottom of the figure, the expression results for mutant 2 and mutant 3, as described previously (15) are summarized for comparative purposes. These expression values have been corrected by a factor of 1.28 to reflect the difference in wild type values obtained here (7.3) versus the values obtained previously (9.4).

**FIG. 8.** GMS assays of promoter fragments. The \( \beta \)-globin promoter fragments containing the CPS sequences (mut 5 + CPS, lane 1; del. CAAT + CPS, lane 2; and mut 7 + CPS, lanes 3–7) were synthesized using polymerase chain reaction and then used as the labeled DNA probe in GMS assays with MEL extracts. The assays were done in the presence of approximately a 100-fold excess of the oligonucleotide shown at the top of each lane. C/EBP-CP1, no competitor added.

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mutant 7 (competes CP1 specifically) the bands at the positions of C/EBP \( \gamma \) and C/EBP \( \beta \) increased (lane 7). Moreover, when the CPS-mutant 7 fragment was competed with the CPS oligonucleotide, the GMS pattern observed exactly coincided with the pattern obtained for CPS-mutant 5 and CPS-deleted CAAT (compare lane 7 to lanes 1 and 2 in Fig. 8), showing that the only extra protein that binds to CPS-mutant 7, relative to these two other promoter fragments, is CP1.
linked H-2K reporter gene (not shown). This suggests that the absence of active C/EBPγ in K562 cells, alone, may not account for the inactivity of the CAAT box in these cells. However, we also found that K562 cells, but not MEL cells, express active LIP. LIP has been shown to be an inhibitor of transcription (21). LIP does not contain transcripational activation domains that are located in the N-terminal half of the larger C/EBPβ proteins (30). In binding to its cognate sequence, LIP will prevent potential transcription activators, including other C/EBP members, from binding there. Therefore, it is likely the combination of not containing active C/EBPγ protein and having LIP that keeps the CAAT box tightly silent in K562 cells. We also cannot rule out the possibility that CP1 is defective in K562 cells (see below).

C/EBPγ May Cooperate with CP1—Our results showed that a CPS oligonucleotide, which binds only to C/EBPγ proteins in MEL cells, was unable to restore activity to the mutant 5 and the deleted CAAT β-globin promoters, but was able to restore wild type levels of activity to the mutant 7 promoter. In terms of factors that normally bind to the wild type CAAT sequence, the deleted CAAT and mutant 5 sequences do not bind to any factors, whereas mutant 7 retains full binding of CP1, but does not bind to any of the other factors in MEL cells (Ref. 15; summarized in Fig. 7). Thus, these expression results suggest the possibility that C/EBPγ cooperates with CP1 to enhance transcription through the β-globin CAAT box. In fact, this interpretation would concur with our previous findings (15). A CAAT box mutation (mutant 2) that retained binding of C/EBPγ (DSFr), C/EBPβ (DSF1), and CP1 stimulated transcription above wild type levels, whereas a mutation (mutant 3) that also retained binding of C/EBPγ and C/EBPβ, but had a greatly reduced affinity for CP1, only stimulated transcription slightly above background (these results are summarized in the bottom part of Fig. 7 for comparison). This again could be explained by the difference in CP1 binding to the two mutants. Note that CP1 binding alone has no stimulatory activity above background (Fig. 7). Thus, it appears that C/EBPγ and CP1 may indeed collaborate. However, this possibility does cause a dilemma. The binding sites for C/EBPγ and CP1 in the β-globin CAAT box overlap each other (15). Hence, it would be expected that the binding of the two proteins is mutually exclusive. Indeed, we have been unable to detect both proteins binding together onto the wild type CAAT box. However, the situation is similar to what has been described for C/EBP factors activating the albumin promoter (31). The C/EBP DNA-binding site is also tightly juxtaposed to a binding site for CP1 in the albumin promoter. Although the binding of both factors is not totally mutually exclusive in this case, C/EBP binding interferes with CP1 binding. Yet, binding of both proteins is again necessary for transcriptional activation (31). Therefore, in some cases C/EBP proteins act in synergism with CP1, even when the factors might interfere with each others binding. If C/EBPγ and CP1 do collaborate to enhance transcription through the β-globin CAAT box, the possibility that both proteins can bind to the CAAT box together under in vivo conditions cannot be excluded. It would also seem possible that C/EBPγ and CP1 are necessary for different steps in the formation of the preinitiation transcription complex and that each protein's function is required at a different time, i.e. the binding of both proteins may be necessary, but may not have to occur simultaneously. Further experiments will be necessary to determine conclusively whether C/EBPγ and CP1 do collaborate at the β-globin CAAT box, and, if so, what the function of each protein is in enhancing β-globin gene expression.

Does the Differential Expression of C/EBP Proteins in MEL and K562 Cells Reflect a Developmental Role?—As K562 cells only express the endogenous α- and γ-globin genes and not the adult stage-specific β-globin gene, they have been considered a model for the embryonic-fetal stages of erythroid development (see Ref. 11, for example). On the other hand, MEL cells are considered a fetal-adult model. Thus, it seems possible that the differential expression of C/EBP factors in K562 cells (active LIP and no active C/EBPγ) versus MEL cells (active C/EBPγ and also active full-length C/EBPβ in induced cells) and the functioning of the β-globin CAAT box only in MEL cells might be related to a developmental specificity. However, it has previously been shown that both C/EBPγ and C/EBPβ are expressed in rat yolk sac and fetal liver, at least at the RNA level (20). This sheds some doubt on the possibility that the differential expression of C/EBP factors in MEL and K562 cells is related to developmental switching of β-globin gene expression.

On the other hand, the differential expression of C/EBP factors has been correlated with cellular differentiation and cell maturation in a variety of systems. For example, the differential expression of three C/EBP members, α, β, and δ, has been directly related to adipocytic differentiation (16, 32). The differential expression of LIP and full-length C/EBPβ has been correlated with liver regeneration. Whereas more LIP is present in actively dividing liver cells, more full-length C/EBP protein is present in quiescent cells and this is directly related to cell growth (33). In this sense, it is important to note that MEL cells and K562 cells also appear to represent different precursors in the erythroid pathway. For example, MEL cells are completely committed to becoming red cells, but this is not the case for K562 cells. If K562 cells are treated with phorbol esters, they lose their erythroid characteristics and induce megakaryocyte markers (34, 35). Moreover, we have obtained initial results that suggest that de-regulated expression of C/EBP factors in K562 cells can alter both erythroid and megakaryocytic differentiation of these cells.2 Thus, we suspect that the differential expression of C/EBP factors in K562 cells versus MEL cells, as well as the inactivity of the β-globin CAAT box in K562 cells, may be related to the different precursor status of these cells.

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