The B Subunit of the CCAAT Box Binding Transcription Factor Complex (CBF/NF-Y) Is Essential for Early Mouse Development and Cell Proliferation

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ABSTRACT

To understand the physiological function of the mammalian heterotrimERIC CCAAT binding factor CBF, also known as NF-Y, we have generated a conditional Cbf-b mouse mutant by introducing loxP sites in the murine Cbf-b/Nf-ya gene. Controlled expression of Cre recombinase deletes the gene in vivo, which leads to a loss of DNA binding by the CBF complex and hence CBF-mediated transcription. Deletion of both Cbf-b alleles causes early embryo lethality, indicating that CBF activity is essential for early mouse development. In primary cultures of mouse embryonic fibroblasts, conditional inactivation of CBF results in a block in cell proliferation and inhibition of S phase or DNA synthesis, which is followed by induction of apoptosis. We conclude that the CBF transcription factor complex is essential for cell proliferation and viability.

INTRODUCTION

The mammalian CCAAT motif-binding factor, CBF (also called nuclear factor-Y or NF-Y) is an evolutionarily conserved transcription factor present from yeast to human. It consists of three different subunits, CBF-A, CBF-B, and CBF-C, which are all required for formation of a specific CBF-DNA complex. Both the CBF-A and CBF-C subunits contain a histone-fold motif and interact with each other to form a CBF-A/CFB-C heterodimer. The A/C heterodimer then interacts with a 21-amino acid stretch in CBF-B to form the heterotrimERIC CBF transcription factor. Thus, the absence of any one of the CBF subunits results in loss of binding of the CBF complex to DNA and CBF-directed transcription (1, 2).

The CCAAT motif is present in promoters of many mammalian genes including genes expressed in specific cell types as well as genes regulated during the cell cycle, such as topoisomerase IIa, cyclin B1, CDC25C, E2F1, CDC2, and thymidine kinase genes (1–6). Even though CBF activity is found to be present in all mammalian tissues, the genes that are regulated by this transcription factor complex in vivo are still unknown. The physiologically relevant target genes, whose transcription is highly dependent on CBF activity, can only be identified using an animal model where endogenous CBF activity can be abrogated in a specific tissue or at a specific developmental stage.

Previously, expression of the dominant negative CBF-B mutant in mouse NIH 3T3 fibroblasts caused the cells to grow slower, with a modest increase in the time required for the cells to complete the S phase of the cell cycle (7). Because transformed cell lines, such as 3T3 fibroblasts, have aberrant expression of many proteins, the effects of dominant negative CBF-B expression in these cells could be masked or accentuated, which would not allow for a clear understanding of the physiological role of CBF activity.

To establish the role of CBF-mediated transcription in vivo, we generated a conditional allele of the gene for the CBF-B subunit, which could be deleted using the Cre-lox system. Using this mouse model, this study clearly demonstrates that CBF-dependent transcription is essential during early mouse development. Conditional deletion of both Cbf-b alleles in primary cultures of MEFs caused a complete block in the progression of the cells into S phase. Subsequently, Cbf-b null cells underwent apoptosis. The results described in this study clearly show that CBF-mediated transcription is required for cell proliferation and viability.

MATERIALS AND METHODS

Construction of the Cbf-b^{floxed} Targeting Vector, Generation of B^{floxed} Allele in ES Cell Clones, and Southern and PCR Analysis of Mouse Tail DNA.

The seventh intron of the murine Cbf-b gene was cloned by amplification of genomic DNA using published sequences (8). The internal probe (Fig. 1A) was used in screening a λ FIXII library comprising SV129 genomic DNA. The unique EcoRV site in the second intron was altered to a BamHI site, and a loxP sequence (1) was cloned at the 3′ end of this restriction site by ligating an oligonucleotide cagctcccgccgctacgtactgtagtattgtatgcataagttccgggtgta (B^{floxed} allele). The neomycin resistance gene, flanked by loxP sites II and III from pPGKneoAloxP (9), was cloned into the unique SphI site in the eighth intron with a BamHI site also introduced at the 3′ end of the 3′ loxP site. The human thymidine kinase gene was cloned 5′ region to the SphI site, as shown in Fig. 1A.

Using published methods, the targeting vector was introduced into ES cells (9). Southern analysis of the BamHI and HindIII-digested genomic DNA, using the 5′ and 3′ external probes, was used to confirm homologous recombination in selected ES clones. Individual ES clones were used to obtain chimeric mice, which were then crossed with C57BL/6 mice to obtain animals that have the B^{floxed} allele.

Genomic DNA from mouse tails, cells, or embryonic tissue was isolated by digestion in a buffer containing 0.5 μg/ml proteinase K, NaCl, EDTA, and SDS at 55°C overnight. PCR primer 1 (gtaagtcaggctccaggg), in intron 2 and 5′ of the EcoRV site of the CBF-B gene, and primer 2 (aggcaaggcagatttaggaag-gcctatacgaagttatcccgggtgttga) (Table 1), in the seventh intron of the murine Cbf-b gene, were used to distinguish between the 200-bp B^{flox} allele and the 250-bp B^{flos} allele. Primer 1 and primer 2 (ggggtgcaggagtcgcag), in intron 8 and 3′ of the SphI site, were used to amplify the 400-bp product to genotype a B^{del} allele.

MEF Cultures. We used 13.5 d.p.c. floxed and B^{flox} embryos to isolate MEFs using previously published procedures (10). MEFs were used at passage 4 and 5 for the experiments.

AdCre Infection of MEFs and Detection of β-Gal Activity in ROSA MEFs.

AdCre was a kind gift from Dr. Frank Graham (McMaster University, Ontario, Canada), and the adenovirus was amplified by the Adenovirus Core Facility at our institute. MEFs (4 × 10^6), plated on 10-cm dishes, were rinsed with PBS containing Mg^{2+} and Ca^{2+}. The appropriate amount of AdCre for a MOI of 10, in 1 ml of PBS, was added to the cells and placed at 37°C for 70 min with rocking every 15 min, after which PBS was removed and replaced with 10 ml of fresh MEF medium.

1 The abbreviations used are: MEF, mouse embryonic fibroblast; ES, embryonic stem cells; d.p.c., days post conception; MOI, multiplicity of infection; β-gal, β-galactosidase; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EEF IG, Mus musculus elongation factor like protein mRNA.
Forty-eight h after infection, mock-infected and AdCre-infected ROSA MEFs were stained for β-gal activity using previously published procedures (9).

Cell Synchronization, BrdUrd Labeling, Immunofluorescence, and Confocal Microscopy. Aphidicolin was added at 2 μg/ml to MEFs plated at 4 × 10^5 cells/10-cm dish in regular MEF medium containing 15% FCS for 40 h. Cells were labeled with 10 μM BrdUrd for 1 h, fixed with paraformaldehyde, and processed for immunofluorescent detection with anti-BrdUrd antibody using a FLUOS kit following the suggested protocol (Roche Molecular Biochemicals). MEFs grown on gelatin-coated coverslips were fixed for 10 min with 1% paraformaldehyde, permeabilized in 0.5% Triton X-100 for 30 min, and blocked in 80 mM PIPES (pH 6.8), 5 mM EGTA, 2 mM MgCl₂ (PEM buffer) containing 1.5% nonfat dry milk. A polyclonal antibody directed against the band and a 250-bp specific restriction fragments in the genomic clone is shown. Restriction sites for BamHI, KpnI (K), Ndel (O), SphI (S), SnaI (S), EcoRV (E), SstI (Sp) are shown. The loxP sites are indicated as I, II, and III. Positions of the 5' and 3' external probe and internal probe and PCR primers 1–3 used in mouse genotyping are shown here. B, restriction map of the Bflox and Bdel alleles generated in vivo from the targeted Bflox+neo+ gene. Positions of BamHI and Ndel restriction sites are shown, which were used to genotype Bflox and Bdel alleles. C, Southern analysis of mouse genotypes. BamHI-digested mouse tail genomic DNAs were used for Southern analysis using both the 5' and 3' middle probes. D, PCR analysis of genomic DNA to detect Bflox and Bdel alleles. PCR primers 1, 2, and 3, which flank the 5' and 3' of the EcoRV site in the Cbf-b genomic locus, amplify a 200-bp Bflox band and a 250-bp Bdel band. E, PCR detection of Bflox allele. Primers 1 and 3 amplify a 400-bp band only when Bdel allele is present. Location of the primers is shown in A.

RESULTS

Generation of a Conditional Bflox Allele. To disrupt the endogenous mouse gene for the CBF-B subunit, we used the Cre recombinase-loxP system (11). The 346-amino acid-long murine CBF-B polypeptide is encoded in nine exons, of which the DNA binding region and the subunit interaction domains are encoded in exons 7 and 8 (8). To create a conditional Cbf-b allele (Bflxed), which would remove all of the functional domains of CBF-B, loxP sites flanking exon 3 and exon 8 were inserted in the targeting vector. Targeted ES clones were used to generate chimeric mice, and mice heterozygous for the targeted Bflox+ targeted Bflox+ allele were obtained. Crosses with CMVCre C57BL6 transgenic strain, in which a cytomegalovirus promoter directed Cre expression during early mouse embryogenesis, generated all three possible recombinant B alleles. Recombination between loxP sites II and III created the Bflxed allele, which left the B gene flanked by two loxP sites for future tissue-specific targeted deletion of the Bflox gene (Fig. 1B). Recombination between loxP sites I and III in the Bflox+neo+ allele created a Bdel allele, which removed both the functional DNA binding and transactivating regions of the protein. Recombination between loxP sites I and II deleted the B sequences and left the neo gene. This Bdelneo+ allele, which was identified by PCR amplification of the neo sequence, was not used.

To identify the different alleles, Southern analysis of BamHI-digested genomic DNA was performed with two probes (Fig. 1C).
The 5’ probe identified the 9-kb B'w (Lanes 1–4) and the 7-kb B’ allele, respectively (Lanes 1–3). In addition, an internal probe, which lies within the sequences targeted for deletion, identified the B'w, B'w, and the B'w allele. Indeed, addition of the neo cassette in the targeted locus introduced a BamHI site in the 3' end of the neo gene, which persists even after the neo sequence is deleted. Hence, BamHI restriction fragments of 5.5, 4.5, and 3.5 kb identified B'w (Lane 3), B'w (Lanes 1–4), and B'w (Lane 2), respectively. Because this probe lies within Cbf-b sequences targeted for deletion, the absence of a band corresponding to a ‘targeted’ allele, as detected by the 5’ probe, indicated a B' allele (Lane 1).

Two sets of PCR amplification from mouse tail DNA were also used for genotyping, as shown in Fig. 1, D and E. PCR primers 1 and 2 amplified a 200-bp B’w band or a 250-bp B’w band (Fig. 1D, Lanes 1–3). Primers 1 and 3 amplify a 400-bp band of the B' allele (Fig. 1E, Lanes 2 and 3).

Heterozygous animals, with one allele of Cbf-b deleted, were normal and fertile. In crosses between heterozygous mutants, no homozygous B' mice were obtained, as early as the 8.5 d.p.c. stage of embryonic development. The distribution of the three genotypes, detected at the specific developmental stages, is shown in Table 1. These results indicate that CBF activity is essential for early mouse embryo development.

**Generation of Cbf-b Null, or B' mice.** The role of CBF-B expression in cell cycle progression was tested in primary cultures of MEFs from heterozygous 13.5 d.p.c. embryos, containing one B' allele and one B' allele, that were infected with adenovirus expressing Cre recombinase (AdCre). Efficiency of virus-mediated delivery of Cre recombinase (Cre) and its subsequent enzymatic activity were tested in MEFs isolated from ROSA26R embryos, where β-gal is expressed after the removal of a stop cassette flanked byloxP sites (11). These B' cells served as a control to rule out nonspecific effects of Cre. Around 80–90% of B' cells were positive for β-gal activity 48 h after AdCre infection at a MOI of 1:10, whereas uninfected cells were negative. For detection of Cre-mediated recombination and deletion of the second Cbf-b allele in B' cells, Southern analysis of NdeI-digested genomic DNA was done, using the 5’ probe (Fig. 2A). The 9-kb band corresponds to the B'w or B'w locus, and the 11.5-kb band corresponds to the B' allele (Fig. 1B). A scan of these autoradiograms indicated that more than two-thirds of the cells in culture were B-null (B' mice) at 72 and 96 h after infection with AdCre. To maximize recombination and increase the percentage of B-null cells in culture, higher MOIs with AdCre were tested. Increasing the level of Cre protein had a deleterious effect on cell growth of the control B' cells, probably due to nonspecific recombination as reported by other investigators (12), and was not used in this study. Because we used the optimal MOI for all our experiments, we consider that any effect in the B' MEFs that we describe here was caused specifically by the deletion of both Cbf-b genes.

Subsequent loss of CBF-B protein at 96 h after viral infection was monitored by immunofluorescence and confocal microscopy. The top row of Fig. 2B shows the presence of CBF-B in all nuclei of B' MEFs infected with AdCre. All nuclei of uninfected B' MEFs (Iox cells) also show the presence of CBF-B (second row of Fig. 2B). The bottom row shows absence of anti-CBF-B staining in six of seven nuclei at 96 h postinfection with AdCre, whereas one nucleus is still positive for CBF (white arrow). These fields, which are representative of many that were examined, reflect the results of the Southern analysis, which indicated that 70% of the B' MEFs had undergone recombination and were null for the Cbf-b gene. Loss of CBF-B protein also caused a marked decrease in CCAAT motif binding activity when tested in DNA binding experiments using nuclear extracts from these MEFs (data not shown). We concluded that the B' allele of Cbf-b was deleted in about 70% of the cells after adenoviral delivery of Cre recombinase, that no CBF-B polypeptide was present in B' null cells at 96 h postinfection, and that CBF-DNA binding activity was lost in this population of cells.

### Table 1 Distribution of embryo genotypes

<table>
<thead>
<tr>
<th>Stage</th>
<th>B wild-type</th>
<th>B heterozygote</th>
<th>B null</th>
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<tr>
<td>Newborn</td>
<td>41</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td>13.5 d.p.c.</td>
<td>8</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>8.5 d.p.c.</td>
<td>6</td>
<td>10</td>
<td>0</td>
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**Decrease in Growth Rate of the B Null Cells in Culture.** At 72, 96, 120, and 144 h postinfection, cells were counted to estimate growth rate in the presence or absence of CBF-B. As shown in Fig. 2, A, in vivo excision of B allele in B MEFs. Genomic DNA was isolated from B' or B MEFs after 72 or 96 h with AdCre. The DNAs were digested with NdeI for Southern analysis with the 5’ probe. B loss of CBF-B protein in AdCre-infected B MEFs. Cells were stained with propidium iodide for DNA. A polyclonal CBF-B antibody was used with a fluorescein-tagged secondary antibody, and the analysis was done by confocal microscopy (×40 or ×63 magnification). CBF-B protein is detected in all nuclei of B' cells infected with AdCre (Wt + AdCre) and of uninfected B' MEFs (Flax + AdCre). CBF-B is detected in one of seven nuclei (indicated by white arrow) of AdCre-infected B MEFs (Flax + AdCre). C, cessation of growth in B MEFs infected with AdCre. Numbers of B' and B MEFs at 0, 72, 96, 120, and 144 h after AdCre infection are shown as a bar graph. Both parts of this figure are representative of six different experiments, which showed similar results.

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**Fig. 2.** A, in vivo excision of B allele in B MEFs. Genomic DNA was isolated from B' or B MEFs after 72 or 96 h with AdCre. The DNAs were digested with NdeI for Southern analysis with the 5’ probe. B loss of CBF-B protein in AdCre-infected B MEFs. Cells were stained with propidium iodide for DNA. A polyclonal CBF-B antibody was used with a fluorescein-tagged secondary antibody, and the analysis was done by confocal microscopy (×40 or ×63 magnification). CBF-B protein is detected in all nuclei of B' cells infected with AdCre (Wt + AdCre) and of uninfected B' MEFs (Flax + AdCre). CBF-B is detected in one of seven nuclei (indicated by white arrow) of AdCre-infected B MEFs (Flax + AdCre). C, cessation of growth in B MEFs infected with AdCre. Numbers of B' and B MEFs at 0, 72, 96, 120, and 144 h after AdCre infection are shown as a bar graph. Both parts of this figure are representative of six different experiments, which showed similar results.
2C, B\textsuperscript{wt/wt} cells continue to increase in number and undergo \sim 3 rounds of doubling in the span of a 144-h culture period. Infection of the B\textsuperscript{wt/wt} cells with AdCre did not cause much difference in their growth rates when compared with that of uninfected cells. However, uninfected B\textsuperscript{flox/del} cells grew slower than uninfected B\textsuperscript{wt/wt} cells. In addition, at 72 h after infection of B\textsuperscript{flox/del} cells, there was no increase in the number of cells in culture. This absence of cell proliferation in the population of infected B\textsuperscript{flox/del} cells (mainly B\textsuperscript{flox/del} cells) at this time in culture indicated a delay or block in the cell cycle. A similar result was observed in multiple experiments using MEF cultures established from four different B\textsuperscript{flox/del} embryos. This showed that complete loss of CBF-B protein in MEFs resulted in growth arrest.

**Analysis of Cell Cycle Progression of B\textsuperscript{wt/wt}, B\textsuperscript{flox/del}, and B\textsuperscript{flox/del} MEFS.** To identify cells in S phase of the cell cycle, infected or uninfected B\textsuperscript{wt/wt} and B\textsuperscript{flox/del} MEFs were BrdUrd-labeled for 1 h and processed for immunohistochemistry (Fig. 3, top panel; Table 2). At 96 h after infection with AdCre, 18% of the B\textsuperscript{wt/wt} nuclei stained positive with the BrdUrd antibody, whereas B\textsuperscript{flox/del} MEFs infected with AdCre had less than 2% of their nuclei positive for BrdUrd labeling. Thus, in the absence of CBF-B protein, there is a marked decrease in the number of cells in S phase. This could be due to a G\textsubscript{1}-S block or an inability to replicate DNA. Interestingly, uninfected B\textsuperscript{flox/del} cells, which proliferate more slowly in culture than wild-type cells, had 12% of cells in S phase compared with 25% of BrdUrd-labeled nuclei in uninfected cells with wild-type levels of CBF-B protein. Thus, heterozygous level of CBF-B protein causes a 50% decrease in S phase-positive MEFs, and B-null cells had an almost complete block in DNA synthesis.

Similarly, at 120 h after infection with AdCre, cells were analyzed for the presence of histone H3 phosphorylation at serine 10, which is a marker for M phase (13). Uninfected B\textsuperscript{wt/wt} cells and uninfected B\textsuperscript{flox/del} cells have 16% and 14% of cells positive for histone H3 phosphorylation (Fig. 3, bottom panel; Table 2). On the other hand, the B\textsuperscript{wt/wt} cells that were infected with AdCre have 12% of nuclei positive for phosphorylated histone H3. Surprisingly, around 7% of nuclei of AdCre-infected B\textsuperscript{flox/del} cells, which are mainly B\textsuperscript{flox/del} cells at this time, are positive for phosphorylated histone H3. In B\textsuperscript{wt/wt} cells infected or uninfected with AdCre, the S phase:M phase ratio is about 1.5, which indicates that there are about one-third more cells in S phase than in M phase. In contrast, in uninfected B\textsuperscript{flox/del} cells, the ratio of S phase:M phase is 0.86, indicating that fewer cells are in S phase as in M phase. Interestingly, after B\textsuperscript{flox/del} cells were infected with AdCre, the ratio of S-phase nuclei: M-phase-positive nuclei drops further to 0.28. Thus, in the population of cells where at least 70% of MEFs have no detectable CBF-B protein, even though there is a further drastic drop in cells able to enter S phase, nuclei still show positive labeling for M phase. This indicates that in the absence of CBF-B, there is an aberrant distribution of cells in specific cell cycle stages. At these times, or later, there is also no apparent increase in cell number in B\textsuperscript{flox/del} MEFs. These results support the view that in the absence of CBF-B, most cells are blocked from either entering into or progressing through S phase. In addition, some cells are also stalled in the M phase of the cell cycle or may be prematurely entering mitosis.

The slower growth of uninfected B\textsuperscript{flox/del} cells and the lower proportion of these cells in S phase compared with B\textsuperscript{wt/wt} cells could be due to CBF-B haploinsufficiency in the primary fibroblasts. To further analyze these differences, aphidicolin was used to arrest the B\textsuperscript{flox/del} and B\textsuperscript{wt/wt} cells at the G\textsubscript{1}-S boundary. After cells were released from the block, entry into S phase was assayed by BrdUrd incorporation. Interestingly, the B\textsuperscript{flox/del} cells had only 4% of cells in S phase, whereas 40% of B\textsuperscript{wt/wt} cells were progressing through the S phase at this time (Table 2). This discrepancy between the B\textsuperscript{flox/del} and the B\textsuperscript{wt/wt} cells demonstrates that the lower level of CBF-B protein compromises the ability of cells to recover after a block in DNA synthesis.

**B\textsuperscript{flox/del} Cells, Infected with AdCre, Undergo Apoptosis.** Nuclei of many B-null cells had a smaller size, showing compaction of their DNA. Other nuclei were large and undergoing abnormal blebbing with formation of micronuclei. These abnormal morphologies suggested that the cells could be undergoing programmed cell death and/or mitotic catastrophe. To further document the hypothesis of apoptosis, at 96, 120, and 144 h after AdCre infection, B\textsuperscript{wt/wt} MEFs and B\textsuperscript{flox/del} cells were analyzed by TUNEL assay. At 96 h after

| Table 2 | Comparison of B\textsuperscript{wt/wt}, B\textsuperscript{flox/del}, and B\textsuperscript{flox/del} MEFS that are in either S phase or M phase, as described in Fig. 3.

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<th>Time (h)</th>
<th>S phase</th>
<th>M phase</th>
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<tr>
<td>96</td>
<td>25</td>
<td>16</td>
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<tr>
<td>120</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>144</td>
<td>12</td>
<td>14</td>
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<td>25</td>
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For each experiment, a total of 500 nuclei were counted to determine percentage of nuclei stained for BrdUrd incorporation or phosphorylated serine 10 of histone 3. In addition, a 40-h aphidicolin treatment was used to arrest uninfected B\textsuperscript{wt/wt} and B\textsuperscript{flox/del} cells at G\textsubscript{1}-S boundary. Six h after removing the block, BrdUrd labeling, immunochemistry, and microscopy were used to count 500 nuclei to estimate the percentage of BrdUrd-positive nuclei. These results were validated in two separate experiments.
infection, when there was already an effect on cell growth in infected B^{floxdel} cells, there were almost no TUNEL-positive cells in either uninfected or AdCre-infected B^{wt/wt} and B^{floxdel} MEFs (data not shown). At 120 and 144 h after infection with AdCre, only 1% of nuclei of B^{wt/wt} cells were positive by TUNEL (Fig. 4). Similarly, only 4% of nuclei of uninfected B^{floxdel} cells were positive for apoptosis. In contrast, at 120 and 144 h after AdCre infection, about 75% of B^{floxdel} nuclei were positive for TUNEL staining (Fig. 4, bottom three rows). The bottom row in Fig. 4 shows a higher magnification view of two nuclei that are TUNEL positive; one is a condensed nucleus, and the other has a normal size. Taken together, these results demonstrate that in the absence of CBF-B, progression through the cell cycle is inhibited, and apoptosis is initiated.

**Increased Expression of Caspase-2 Gene in CBF-B-Null Cells.**

Because B^{floxdel} cells had a block in cell proliferation and were undergoing apoptosis, we studied changes in gene expression using cell cycle-related and apoptosis-related cDNA arrays. Total RNA was isolated at 112 h after infection with AdCre from B^{wt/wt} and B^{floxdel} MEFs and reverse transcribed, and the cDNAs were hybridized to filters containing sequences of 39 apoptosis-related genes.

Expression level of the proapoptotic gene, initiator caspase-2, was increased 2-fold in CBF-B-null cells but unchanged in B^{wt/wt} cells either infected or uninfected with AdCre (Fig. 5) in two separate experiments. In contrast, expression of all other genes present in the apoptosis-related cDNA array was not significantly changed in all four sets of RNA samples. The increase in caspase-2 mRNA was further substantiated as a 3-fold change using real-time PCR analysis (data not shown).

**DISCUSSION**

Expression of Cre recombinase during early mouse embryogenesis resulted in deletion of a Cbf-b^{flox} allele in vivo. Heterozygous mice, with deletion of one Cbf-b allele, developed normally, similar to mice with both wild-type Cbf-b alleles. However, when heterozygous mice were bred, no Cbf-b-null mouse embryos were detected, as early as the 8.5 d.p.c. stage, indicating that CBF-B is required for early embryonic development. Expression of Cre in CBF-B heterozygous mutant MEFs carrying one functional B^{flox} allele created B-null cells, which were unable to proliferate in culture and eventually underwent apoptosis. Inhibition of cell growth correlated with the deletion of the remaining Cbf-b allele and the loss of CBF-B polypeptide in the nucleus. In the conditions used in our experiments, there was no significant alteration of cell growth in B^{wt/wt} MEFs. Thus deletion of both Cbf-b alleles caused a specific block in cell proliferation and caused cell death. We hypothesize that the early embryo lethality in mice is also due to an inability of cells to proliferate and survive.

We have consistently observed that the growth rate of heterozygous B^{floxdel} fibroblast cells is significantly lower than that of B^{wt/wt} cells in culture. This is further supported by the observation that the proportion of these cells in S phase is clearly lower than that of the B^{wt/wt} cells. Hence, in primary cultures of fibroblast cells, loss of half the amount of CBF-B decreases the number of cells in S phase. Furthermore, B^{floxdel} fibroblast cells are sensitive to cell cycle arrest by aphidicolin. On removal of aphidicolin treatment, B^{wt/wt} cells reentered S phase, but B^{floxdel} cells were unable to synthesize DNA, as ascertained by BrdUrd incorporation. Aphidicolin inhibits DNA polymerase α, δ, and ε and causes stalled replication forks, and removal of aphidicolin allows cells to restart DNA synthesis. Prolonged stalling of replication in the presence of aphidicolin causes the loss of specific MCM proteins from chromatin in Xenopus cells. These proteins are required for reinitiation of replication and are...
regulated stringently to avoid multiple replications of DNA within a single cell cycle (14). We hypothesize that loss of half the amount of CBF-B protein, in a specific cell type such as fibroblasts, might be responsible for the expression of lower amounts of proteins required to reinitiate replication.

Analysis of MEFs by TUNEL assay showed that loss of CBF-B induces apoptotic cell death. The time of cell death occurred after the arrest of the cell cycle because almost no cells were TUNEL positive at 96 h after AdCre infection, when entry into or progression through S phase was already inhibited. However at 144 h after AdCre infection, the majority of MEFs lacking CBF-B were positive for TUNEL staining. Thus CBF is a survival factor for mammalian cells and is required for cell cycle progression. By analyzing the expression of genes identified previously that lead to or prevent apoptosis, we found that expression of caspase-2 mRNA was increased in the absence of CBF-B but that the expression of other genes present in the array was unchanged in the time interval between the S-phase inhibition and the detection of TUNEL-positive cells. Examination of the proximal promoter region of both the mouse and human caspase-2 gene shows the presence of two CCAAT motifs within 250 bp of the transcription start site. Conservation of the putative CBF binding sites indicates that transcription of caspase-2 mRNA might be directly regulated by CBF. The increase in caspase-2 mRNA in CBFFull-null cells suggests that the transcription of this gene could be negatively regulated by CBF, which would require further experimental analysis. Recently, an increase in the expression of caspase-2 mRNA, specifically in response to DNA damage, was reported in a human cell line (15). Caspase-2, which is one of the initiator caspases, triggers the processing of downstream effector caspases, is localized mainly in the nucleus, is autoactivated by oligomerization and binding to its adapter protein RAIDD, and has been shown to be transcriptionally up-regulated in response to certain hormonal conditions or stress (15–17). Expression levels of other initiator caspases, which are localized in other specific nonnuclear compartments such as the endoplasmatic reticulum, Golgi, and mitochondria, were not increased. We hypothesize that inhibition of the S phase or premature entry into the mitotic phase in B-null cells could cause DNA damage and result in the induction of caspase-2 mRNA.

In summary, our study establishes that an important in vivo function of CBF is to control cell proliferation, particularly entry in and progression through the S phase of the cell cycle. Although the mechanism by which CBFF binds cell cycle progression remains to be clarified, recent preliminary RNA expression analysis (data not shown) indicates the decreased expression of the Cullin family of genes as well as cyclin A2 in Cbf-B-null cells; these genes are important for progression from G1 to S phase of the cell cycle (18, 19). This analysis suggests that inactivation of CBFF results in changes in the expression of multiple genes. Future experiments will identify CBF-dependent genes that are important for cell proliferation and early embryogenesis.

Our mouse model will be useful in determining whether other sites of in vivo proliferation, such as skin, hair, and regenerating livers, are dependent on CBFF-mediated transcription. In addition, by using an inducible Cre recombinase and a mouse model for tumorigenesis, we should be able to test whether tumor cells require functional CBF for survival.

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