rel/NF-κB Nuclear Complexes That Bind kB Sites in the Murine c-rel Promoter Are Required for Constitutive c-rel Transcription in B-Cells

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Abstract

The c-rel protooncogene, a member of a transcription factor family that includes NF-κB, displays a complex pattern of gene expression. To understand the basis of this expression, the regulatory region upstream of the murine c-rel transcription start sites has been cloned and characterized. Transcription of the murine c-rel gene initiates at multiple sites downstream of a GC-rich region conserved in the chicken c-rel promoter. This conserved region contains consensus transcription factor binding sites for SP-1 and NF-κB (κB3 site) and is sufficient for basal expression in Jurkat T-cells. In contrast, two additional NF-κB-like sites (κB1 and κB2) and an octamer consensus binding site, all located upstream of the conserved region, are required for expression of promoter-reporter gene constructs in the B-cell line I29B. NF-κB sites κB1 and κB3 bind p50/65 and p50 homodimers, whereas κB2 binds a distinct complex. The conserved octamer site, although only able to bind Oct1 and Oct2 with low affinity, appears to overlap with a binding site for a novel protein(s) expressed in I29B cells. Cotransfection studies show that p75-c-rel and a carboxyl-terminal truncated c-rel protein that lacks the known trans-activating domain both up-regulate the c-rel promoter in I29B cells via a mechanism independent of the NF-κB motifs, whereas a mutant c-rel protein lacking the DNA binding domain has no effect. Together, these findings suggest that, in this B-cell line, trans-activation of the c-rel promoter by rel proteins is via an indirect mechanism.

Introduction

The c-rel gene was first identified as the cellular homologue of v-rel, the oncogene carried by Rev-T, an acutely transforming avian retrovirus that induces a rapid B-lymphoid leukemia in galliform birds. Cloning of the cDNAs encoding the p50 (p105) and p65 subunits of the NF-κB transcription factor revealed that both proteins share a region of homology with the c-rel protooncogene, its transduced viral counterpart, v-rel, and the Drosophila morphogen, Dorsal. Two further proteins which share this homology, p49 (p100) (1, 2) and reB (3, 4) have been identified. This highly conserved rel domain comprises a region of approximately 300 amino acids located within the amino-terminal half of these proteins and has sequences important for DNA binding, dimerization, and nuclear localization (5). In contrast to the conserved amino-terminal region, the carboxyl termini of c-rel, p65, and reB are completely divergent (3, 6, 7). Transcriptional trans-activating domains have been localized to these regions of p65 and p75c-rel (8, 9). The p50 and p49 proteins consist almost entirely of the rel homology domain. Although both are encompassed within the amino terminus of large precursor molecules (p105 and p100, respectively (1, 2, 10, 11)), only p50 has been formally shown to arise from the p105 precursor by proteolytic cleavage.

The p50/p65 combination of subunits (the classic NF-κB complex) is the best characterized complex of rel-related proteins, the trans-activating properties of which are well established (reviewed in Ref. 12). The p50 homodimer, (p50)2, has been shown to be a constitutive nuclear factor in a variety of cell types, and DNA binding of (p50)2 has been associated with the basal transcription of a number of genes (13–15). Although many other heterodimeric and homodimeric combinations of rel family members have been shown to form in vitro, only some of these have been identified in vivo (16, 17). The precise effect of each complex on gene expression is likely to be determined by a number of influences, including posttranslational modification, the presence of other transcription factors, and the affinity of the complex for the particular κB site (12).

One regulatory mechanism common to rel-related proteins is that these transcription factors are retained in the cytoplasm as inactive complexes by a family of inhibitor (IκB) proteins. A wide variety of stimulatory signals, including growth factors and mitogenic agents, lead to inactivation of the inhibitors, which allows translocation of these activated rel/NF-κB complexes to the nucleus (12). Expression of rel family members has also been shown to be controlled at the level of transcription (7, 18, 19). Levels of c-rel mRNA vary in a differentiation stage-specific manner in cells of the B lineage, with highest constitutive levels confined to B-cell lines expressing surface IγM (20). In other cell types, such as T-cells and fibroblasts, constitutive c-rel expression is very low and can be induced transiently (21, 22). The stimulation of c-rel and p105 transcription by signals known to increase the level of active rel family protein complexes in the

becco's modified Eagle's medium; GST, glutathione S-transferase; Hepes, 4-
(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction.
nucleus raises the possibility that expression of these genes may in part be through autoregulation. Consistent with this suggestion is the finding that c-re sites have been identified in the promoters of the human p105 and chicken c-rel genes that can bind rel/NF-kB complexes (17, 23, 24), although the precise role of these sites in gene expression remains to be determined.

Restriction of high constitutive c-rel expression largely to cells of the B-lymphoid lineage (20), coupled with B-cell-specific transformation induced by the v-rel-bearing retrovirus Rev-T (25), suggests that c-rel may play a key role in B-cell growth and differentiation. Analysis of murine c-rel promoter activity in a B-cell line expressing high levels of endogenous c-rel indicates that constitutive transcription is under complex regulation and that c-rel is able to modulate its own expression.

Results
Molecular Characterization of the Murine c-rel Promoter. The sequence of the 1.4-kb NcoI genomic fragment from Amc-re11, which lies directly 5' of the c-rel initiation codon, is shown in Fig. 1. Inspection of the genomic sequence upstream of the region corresponding to the 5' untranslated region of the murine c-rel cDNA clone, Amc-re1.2 (5' of nucleotide −26 (Fig. 1)), revealed no TATA or CCAAT boxes, features common to promoters that initiate at a unique site.

To ensure that the start sites of murine c-rel transcription were encompassed within the sequence shown in Fig. 1, RNase protection analysis was performed on total cellular RNA samples isolated from the B-cell lymphoma L298, a murine NIH 3T3 fibroblasts stably transfected with FCS and cycloheximide, and splenic B-cells treated with LPS. This analysis, shown in Fig. 2A, revealed multiple start sites of c-rel transcription in all cell types tested. Primer extension analysis of RNA from L298 (Fig. 2B) confirmed the heterogeneous nature of c-rel transcription initiation. Whereas eight major transcription start sites are detected in the L298 cell line (Fig. 2A, Lane 4; denoted 1–8) which together span a region of approximately 100 bp, not all of these sites are utilized in serum-stimulated NIH 3T3 fibroblasts or LPS-treated splenic B-cells (Lanes 5 and 6, respectively). These data indicate that the sites of c-rel transcription initiation may differ according to cell type and the stimulus used to induce c-rel expression. We have arbitrarily designated the first prominent transcription start site common to all cell types tested (site 2 in Fig. 2A) as 0 within the genomic sequence in Fig. 1. An apparent additional site of transcription initiation common to all cell types is revealed by RNase protection analysis when autoradiographs are exposed for prolonged periods (data not shown; 1' in Fig. 2A) but could not be detected by primer extension mapping. The basis of our inability to detect a start site in the locality of 1' (approximately −110 in Fig. 1) by primer extension analysis is not known but may indicate that the high GC content within this region prevents efficient read-through by reverse transcriptase.

Analysis of the sequence 5' of transcription start site 2 (position 0 in Fig. 1) shows that it is highly G-C rich (approximately 77%) over a 300-bp region. Alignment of the murine c-rel promoter sequence with that of the chicken c-rel promoter (23, 24) reveals a 158-bp region of 58% identity encompassed by the G-C-rich domain (underlined in Fig. 1). Present within this region of homology lie putative binding sites for NF-kB and SP-1. The NF-kB site, GGAATCCC, at position −68 to −59 (kB3 in Fig. 1) is identical to one of two NF-kB sites found in the angiotensinogen promoter (26, 27). Two SP-1 sites directly abut each other at nucleotides −114 to −101, whereas overlapping SP-1 sites at nucleotides −29 to −20 map directly downstream of the c-rel homology region.

Additional putative transcription factor binding sites are located upstream of the region homologous with the chicken c-rel promoter, including two additional NF-kB motifs and an octamer binding site. The 5'-most NF-kB site (nucleotides −411 to −402; kB1 in Fig. 1) resembles the NF-kB element within the granulocyte/macrophage-colony-stimulating factor promoter (28), the site located at nucleotides −260 to −251 (kB2 in Fig. 1) most closely resembles the NF-kB motif within the interleukin 2 promoter (29), whereas a consensus octamer binding site [OCT in Fig. 1 (30)] is located at −438 to −431. Numerous other transcription factor consensus binding sites have been identified in the c-rel 5' flanking sequence (see legend to Fig. 1).
Expression of the c-rel Promoter in B-Cells Is Regulated by Multiple Independent Elements. In order to identify regulatory elements necessary for the constitutive expression of c-rel in mature B-cells, a series of fragments derived from the upstream region of the c-rel gene were inserted 5′ of the CAT gene in the promoterless reporter plasmid, pBLCAT3 (here denoted pCAT3) (31). These constructs were transfected transiently into a clonal variant of the murine B-cell lymphoma 129 (129B) (32), a cell line previously shown to transcribe c-rel constitutively (22). Transfection of a plasmid (HP-cat; Fig. 3B, Lane 2) containing 1154 bp of the genomic sequence, extending 880 bp upstream of the major transcription initiation sites, revealed moderate promoter activity approximately 19-fold higher than cell lysates transfected with the parental CAT vector (pCAT3; Lane 1). Interestingly, a 5′ truncation of 240 bp, represented by the RP-cat plasmid (Lane 3) was consistently found to be more active in 129B cells than HP-cat, indicating that a site(s) may exist between HindIII (−880) and RsaI (−640) that is able to exert a negative influence on the activity of the murine c-rel promoter in 129B cells. Subsequent truncations, however, demonstrate a markedly reduced promoter activity relative to the HP-cat construct; the TP-cat plasmid (nts −399 to +274; Lane 4) is 4-fold less active than HP-cat, whereas SP-cat (nts −202 to +274; Lane 5) has virtually no CAT activity.

Constitutive c-rel expression is highest in murine B-cells, whereas in all other cell types, it varies from moderate to undetectable (20). To determine whether this observed difference could be due to alternate regulation of the c-rel promoter in different cell types, the constructs containing 5′ serial truncations of the murine c-rel promoter were transfected into the human T-cell line Jurkat, shown previously to express moderate levels of c-rel mRNA (33). These results (Fig. 3C) show that all of the truncations have equivalent promoter activity in Jurkat cells. This finding is similar to studies using chicken c-rel promoter-reporter gene constructs transfected into an avian T-cell line, MSB1, and primary chicken embryonic fibroblasts, where only approximately 200 nucleotides upstream of the major transcriptional start sites were required for full transcriptional activity (23, 24). Furthermore, the addition of PMA, previously shown to up-regulate c-rel transcription in T-cells and fibroblasts (21, 22), induced a modest increase in promoter activity (approximately 2-fold) of all serial truncations, indicating that a site(s) able to confer PMA-mediated up-regulation of promoter activity in Jurkat cells resides within 200 bp of the major transcriptional start site. The data derived from these 5′ serial truncations demonstrate that the regulation of murine c-rel may differ between B- and T-cells and that elements located between nucleotides −640 and −202 play a major role in the positive regulation of c-rel promoter activity in the 129B cell line.

Scrutiny of the nucleotide sequence between −640 and the transcription start sites for regulatory elements that have been implicated in lymphoid gene regulation revealed the presence of a consensus octamer (Oct) (30) and three potential NFκB (34) sites. To determine the role, if any, of these sites in regulating c-rel expression in B-cells, PstI/HpaI-cat constructs (see “Materials and Methods”) containing specific mutations in the individual sites were introduced by transient transfection into the 129B cell line, and the promoter activity was compared relative to unmutated PstI/HpaI-cat plasmid. These experiments, presented in Fig. 3D, demonstrate the positive regulatory roles that the octamer and NFκB sites play in c-rel promoter expression in the 129B B-cell line. Whereas disruption of any of these individual sites resulted in an approximate 2-fold reduction of activity (Fig. 3D, Lanes 3–6), the loss of a functional octamer sequence plus one of the NFκB sites (Fig. 3D, Lanes 7–9) resulted in a greater loss of promoter activity (8-fold) than the additive decrease of the single mutant constructs alone. Although these studies demonstrate that all three NFκB-like sites within the murine c-rel promoter contribute to
transcriptional activity in B-cells, minor but reproducible differences between the individual sites have been observed, with the functional loss of the kb3 site consistently resulting in the most significant loss of promoter activity.

The Murine c-rel Promoter Contains Binding Sites for rel/NF-xB Family Members. The protein-binding properties of the NF-xB-like sites in the c-rel promoter (Fig. 4A), were investigated using electrophoretic mobility shift assays. When probes containing either the kb1 or kb3 sites were incubated with I29B nuclear extracts, two complexes of identical mobility to those formed when I29B nuclear extract was incubated with the NF-xB site from the immunoglobulin light chain enhancer (kb2) were observed (Fig. 4B). A probe containing the kb2 site formed a complex of lower mobility (Fig. 4B). The upper complex seen with kb2, kb1, and kb3 has a mobility identical to that of an LPS-inducible complex in the pre-B-cell lymphoma 70Z/3 (data not shown) and is presumed to be the p50/p65 heterodimer. kb2 and kb3 bind approximately equal amounts of this complex, whereas kb1 binding is weaker, suggesting that it has a lower affinity. The relative mobility of the lower complex suggests that it is a dimer of p50/p50. kb2 binds less of this complex than does kb1 or kb3, suggesting that the latter have a higher affinity. This is consistent with previous work, in which kb2 has been shown to have a relatively low affinity for (p50)p50 (35-37).

These proposed affinities are supported by competition analysis against kb2, presented in Fig. 4C. The addition of an excess of unlabeled kb1 preferentially reduces the formation...
of the (p50)_2 complex, kB3 competes equally well for (p50)_2 and p50/p65 complex formation, whereas kB1 preferentially reduces the formation of p50/p65. An excess of unlabeled kB2 or oligonucleotides containing mutations of the kB1, kB2, or kB3 sites (see Fig. 4A) showed no competition for (p50)_2 or p50/p65 at 200-fold excess (Fig. 4C, and data not shown). Similar results were obtained using these competitor DNAs against kB1 and kB3 as probes (data not shown).

That these complexes contain p50 was confirmed by the use of antibodies specific for p50 (Ref. 38; Fig. 4D). Prior incubation of I29B extract with anti-p50 but not an Oct2-specific antibody ablated binding of both the proposed p50/p65 and p50 homodimer complexes to kB1, kB2, and kB3. Furthermore, p65-specific antibodies ablated the upper complex but had no effect on the lower complex that bound to kB1 and kB3 (data not shown). Together, these data suggest that kB1 and kB3 are relatively high-affinity sites for (p50)_2, and kB3 is also a relatively good binding site for p50/p65.

The kB2 probe specifically bound to a complex from I29B nuclear extract that was not competed by kB1, kB2, or kB3, although binding was abolished by mutations within the kB2 site (Fig. 4B, and data not shown). This complex was only formed when the amount of poly(dII-C) used in the assay was reduced from 0.5 μg to 0.1 μg or when the salt concentration was reduced from 100 mM to 40 mM. Formation of this complex was unaffected by p50-specific antisera (data not shown). Furthermore, neither in vitro translated p49 nor carboxyl-terminally truncated p65 and c-rel proteins synthesized in Escherichia coli bound to kB2 (data not shown). These data, together with the inability of kB2 to compete for the (p50)_2 and p50/p65 complexes (Fig. 4C), suggest that the complex binding to kB2 may comprise proteins distinct from p50, p65, p49, or c-rel.

**Binding Properties of the Octamer Sequence in the c-rel Promoter.** In addition to the functional role of the three NF-kB sites in the c-rel promoter, a consensus sequence for octamer-binding proteins, located 26 bp upstream of kB1, was shown to contribute to c-rel promoter activity. To isolate the octamer sequence on a convenient restriction fragment for use in gel shift assays, a single base change was introduced at position -476 (Fig. 1) to create a PstI site. The kB1 site within the 88-bp PstI/Smal fragment was mutated to abolish (p50)_2 binding (clone Octc), and this clone was then further mutated in the octamer sequence to generate the subclone Octc (Fig. 5A). As Octc2 is an octamer-binding protein primarily expressed in B-cells, we initially chose to focus our study on Octc2 binding to the octomer consensus site in the c-rel promoter.

As a control for the DNA binding studies, the octamer sequence from the immunoglobulin heavy chain enhancer (OctH), which binds both Oct1 and Oct2 proteins from I29B nuclear extracts, was used (Fig. 5B, Lane 1). Several complexes from I29B extracts bind to Oct (Fig. 5B, Lane 3). Binding of these complexes is greatly diminished, although not completely abolished by mutation within the octamer sequence (Lane 7). However, surprisingly, these Oct1-bound complexes were unaffected by competition with the Oct1 sequence (Lanes 4 and 8). In contrast, this level of Oct1 competition abolished binding of Oct1 and Oct2 to Octc2 (Lane 2). Complexes bound to Oct, could be competed by Oct, but only slightly by Octc. This, together with the re-
duced binding to Octm, indicates that the complexes binding to Oct, require the intact octamer sequence for binding.

Antibodies specific for the Oct1 protein (39) or Oct2 protein (40) were used to further examine whether any of the complexes bound to Oct, contain Oct1 or Oct2. As expected, these antibodies specifically abolished binding of Oct1 or Oct2 to Octm (Fig. 5C, Lanes 2 and 4, respectively). However, addition of either antibody has no effect on the binding of complexes to Oct, (Fig. 5C, Lanes 6 and 8), confirming that these complexes do not contain Oct1 or Oct2.

Although no evidence of Oct2 binding to Oct, was found, it was unclear whether this sequence was incapable of binding Oct2. To answer this question, a defined source of Oct2 was obtained by transiently transfecting COS cells with an expression vector containing a human Oct2 cDNA. Oct2 binds two complexes, the upper being endogenous Oct1 and the lower exogenous Oct2 (Fig. 5D, upper panel, Lane 1, and data not shown). Octm competes out both complexes. Oct, shows some competition for both Oct1 and Oct2, whereas Octm shows none. Thus, the octamer sequence in Oct, is capable of binding both Oct1 and Oct2 but with an apparently lower affinity than the Octm sequence. Binding of Oct2 to Oct, was confirmed when Oct, was used as a probe (Fig. 5D, lower panel). Note that in Lane 1, one of the endogenous COS complexes (lower band in Octm competition lanes) merges into the exogenous Oct2 band (lower band in Oct, competition lanes). As above, Octm abolished binding of exogenous Oct2, whereas Oct, reduced it, and Octm had no effect. In addition, Oct, was able to bind purified Oct1 protein (data not shown).

Thus, it is clear that Oct, has a relatively low affinity for Oct1 and Oct2. The absence of Oct1 and Oct2 binding to Oct, in I29B extracts is probably due to a combination of the low affinity of this site and the presence of other competing endogenous complexes. Our inability to detect Oct1 or Oct2 binding to Oct, in this assay, however, does not rule out the possibility that a low but functionally significant level of octamer protein binding to Oct, occurs in I29B cells.

The Ability of c-rel Proteins to Up-Regulate Expression of the Murine c-rel Promoter in B-Cells Is Independent of the Carboxyl-Terminal Trans-activation Domain. In transient transfection assays, both c-rel and v-rel-encoded proteins are able to modulate the expression of certain promoters that contain NF-κB sites (41, 42). To determine whether p75c-rel is able to regulate the expression of the murine c-rel promoter in B-cells, the I29B cell line was cotransfected with the constructs HP-cat, RP-cat, TP-cat, or SP-cat, and a murine c-rel expression vector (pDAMP56 c-rel). As shown in Fig. 6A, cotransfection of the c-rel expression vector with these reporter constructs in all cases resulted in an up-regulation of promoter activity (approximately 3-4-fold) compared to the promoter reporter plasmids alone. This indicated that c-rel-mediated trans-activation was attributable in part to element(s) residing within the SalI/PstI genomic fragment, which extends only 202 nucleotides upstream of the major transcriptional start site. Cotransfection of the promoterless plasmid pCAT3 and expression vectors for either c-rel or a carboxyl-terminal-truncated c-rel protein did not up-regulate CAT activity relative to that observed with the parental expression plasmids (pDAMP56 and pcDNA-1; data
not shown), confirming that the observed trans-activation is occurring via sequence(s) in the c-rel 5' flanking region and not the reporter plasmid.

Interestingly, p75<sup>−/−</sup> up-regulated transcription to the same degree from plasmids containing the wild-type c-rel 5' flanking sequence or any of one of the mutant NF-kB sites (Fig. 6B). To address the possibility that p75<sup>−/−</sup> was acting through more than one of the three NF-kB sites, cotransfection experiments were conducted with the modified HP-cat plasmid (PstI<sub>c</sub>HP-cat) containing mutations at all three NF-kB sites (kB1; kB2; kB3<sub>C</sub>HP-cat). Although the triple mutant exhibited virtually no promoter activity, it was reproducibly enhanced by cotransfection with c-rel (Fig. 6B; compare Lanes 10 and 11), indicating that in 129B, trans-activation by p75<sup>−/−</sup> does not occur through any of the three functional NF-kB sites located within the murine c-rel promoter.

Since c-rel trans-activation was attributable to sequences within the 476-bp SstI-PstI fragment, the ability of this fragment to bind c-rel protein was assessed. These results, shown in Fig. 6D, indicate that only a 130-bp region (nts -102 to +23) containing the kB3 site bound to c-rel homodimers (Lane 4), and this binding was dependent on the intact kB3 site (Lanes 5 and 8). Both kB1 and kB2 failed to bind c-rel protein (Lanes 6 and 7). The possibility that the c-rel protein may be part of a transcriptional complex but is not directly involved in DNA binding was addressed by using antibodies specific for the c-rel protein in the gel shift assays. Antibodies specific for the amino- and carboxyl-terminal regions of p75<sup>−/−</sup> were unable to ablate or supershift any nuclear complex isolated from untreated or c-rel-transfected 129B cells that bound to sequences within the SstI-PstI fragment (data not shown). The absence of detectable p75<sup>−/−</sup> in 129B nuclear complexes that bind to kB3 could be due to competition by (p50)<sub>2</sub> and p50/p65, which bind this site with high affinity.

![Fig. 6](image)

**Fig. 6.** The murine c-rel promoter is trans-activated by c-rel proteins in the B-cell line, 129B. A, the murine B-cell line 129B was transfected with 10 µg of the indicated c-rel CAT construct and 30 µg of either the pDAMP56 expression plasmid containing no insert (−; Lanes 1, 3, 5, and 7) or the c-rel expression plasmid, pDAMP56c-rel (+; Lanes 2, 4, 6, and 8). Lanes 4 and 2, HP-cat; Lanes 3 and 4, RP-cat; Lanes 5 and 6, TP-cat, and Lanes 7 and 8, SP-cat. B, mutation of the three consensus NF-kB sites (kB1–3) in the murine c-rel promoter does not affect trans-activation by c-rel in 129B. Ten µg of the plasmid PstI<sub>c</sub>HP-cat and derivatives containing kB1<sub>m</sub>, kB2<sub>m</sub>, kB3<sub>m</sub>, or all three sites mutated simultaneously were cotransfected with 30 µg of either the pDAMP56 plasmid (−; Lanes 1, 2, 4, 6, 8, and 10) or the pDAMP56c-rel plasmid (+; Lanes 3, 5, 7, 9, and 11) into 129B cells, and CAT activity was determined as described in "Materials and Methods." Lane 1, pCAT3; Lanes 2 and 3, PstI<sub>c</sub>HP-cat; Lanes 4 and 5, kB1<sub>m</sub>HP-cat; Lanes 6 and 7, kB2<sub>m</sub>HP-cat; Lanes 8 and 9, kB3<sub>m</sub>HP-cat; and Lanes 10 and 11, kB1:2:3<sub>m</sub>HP-cat. C, a carboxyl-terminally truncated c-rel protein can trans-activate the c-rel promoter in the 129B cell line. Ten µg of the plasmid PstI<sub>c</sub>HP-cat (Lanes 2, 4, 6, 8, and 9) and its derivative kb1:2:3<sub>m</sub>HP-cat in which all 3 kB sites have been mutated (Lanes 5–7) were cotransfected with 30 µg of either pDAMP56 plasmid (−; Lanes 2, 5, and 8), pDAMP56 c-rel (−; Lanes 3 and 6), pcDNA1-c-rel<sub>3</sub> (−; Lanes 4 and 7), or pcDNA1-c-rel<sub>3</sub> (−; Lane 9) into the 129B B-cell lymphoma line. Equivalent levels of the wild-type and mutant rel proteins were expressed from either the DAMP56 or pcDNA1 vectors (not shown). It should be noted that the promoter activity of the constructs in these cotransfection assays is significantly reduced compared with those signals displayed in Fig. 3B due to a decreased transfection efficiency, the result of the large amount of plasmid DNA required for these transfections. Average percentage acetylation, which is presented below Panels A–C, was determined as described in legend to Fig. 3, B, binding to Fig. 3B, binding to GST-c-rel fusion protein to probe spanning the SstI-PstI region of the c-rel promoter. Lane 1, SstI-Smal (nts −202 to −102); Lane 2, SpeI-Smal (nts +23 to +102); Lane 3, Smal-PstI (nts +102 to +274); Lane 4, Smal-Spel (nts −102 to +23; note that this fragment contains the kB1 site); Lane 5, SmaI-Spel with the kB3 site mutated; Lane 6, kB1 oligonucleotide; Lane 7, kB2 oligonucleotide; Lane 8, kB3 oligonucleotide. Lanes 1–3 were exposed for 1 week; Lanes 4–8 were exposed overnight. 
To further explore the mechanism by which c-rel up-regulated the expression of its own promoter, several mutants of the c-rel protein were constructed for use in the trans-activation assays. c-rel proteins that lack the carboxyterminal trans-activation domain are able to repress NF-kB-regulated gene expression in T-cells and fibroblasts (24, 42). To determine whether a c-rel protein that lacks the carboxyterminal trans-activation domain is able to repress the murine c-rel promoter in B-cells, cotransfection experiments with c-rel promoter-reporter constructs were performed utilizing a murine c-rel expression plasmid, pcDNAc-re/CΔ, in which the carboxy-terminal amino acids 404–588, encompassing the trans-activation domain of the c-rel protein (9), were removed. These data, presented in Fig. 6C (Lanes 3 and 7), clearly show that c-rel protein lacking the carboxyterminal trans-activation domain (9) is able to up-regulate expression of the murine c-rel promoter in B-cells to the same extent as p75c-rel (Lanes 3 and 6) via a mechanism independent of the three NF-kB sites in the promoter (Lane 7). In contrast, a c-rel protein lacking amino acids 21–135 (c-rel NΔ), which are crucial for DNA binding in vitro, but able to dimerize with wild-type c-rel, p105, or p50 protein5 had no effect on c-rel promoter activity (Fig. 6C, Lanes 8 and 9). Taken together, the DNA binding studies and transfection analysis indicate that, although c-rel-mediated transactivation requires the DNA binding domain of the c-rel protein, and c-rel homodimers can bind to KB3, trans-activation is independent of this site and appears to occur by an indirect mechanism which does not require the carboxyterminal trans-activation domain of p75c-rel.

Discussion

The diverse pattern of c-rel expression induced by a wide variety of stimuli in different cell types (20–22) points to complex transcriptional control. Here, we report the molecular characterization of the murine c-rel promoter and establish that constitutive expression in a cell line representative of a mature B-cell is under the control of multiple regulatory elements that include binding sites for rel/NF-kB proteins.

The murine c-rel promoter, like the chicken homologue (23, 24), belongs to that class of GC-rich eukaryotic promoters characterized by heterogeneous transcription initiation. The eight major transcription start sites identified in this study, which together span approximately 100 bp, overlap part of a region that shares significant homology with the chicken c-rel promoter (58% over approximately 160 bp). A prominent feature of this conserved region is a NF-kB element, the location of which is conserved between the promoters of these two species. The selective use of particular c-rel transcription start sites in different cell types or when expression is induced by different stimuli is also a feature common to the chicken (24) and murine promoters. Although the mechanism and reason for alternative c-rel transcription initiation remain to be determined, they may involve recruitment of transcription factors that are restricted to particular cell types or induced by certain stimuli.

The transfection studies presented here show that regulatory elements located within 200 bp of the first major start site of transcription are sufficient for basal promoter activity and contribute to PMA inducibility in the Jurkat T-cell line.

This is similar to the chicken c-rel promoter, in which 170 bp of sequence flanking the transcription initiation sites were sufficient for basal promoter function in embryonic fibroblasts and an avian T-cell line (23, 24). In the I29B cell line, additional elements contribute to constitutive c-rel promoter activity, including an octamer and two NF-kB sites. Although the individual contribution of the octamer and three NF-kB sites to promoter activity is minor, loss of two or more of these elements results in a dramatic reduction in c-rel promoter activity in I29B cells. Whereas these data indicate that the collective contribution of these elements is required for the moderately high constitutive promoter activity detected in this B-cell lymphoma, it is possible that other as yet unidentified regulatory sequences contribute to c-rel promoter function. To date, transfection analysis of murine c-rel promoter activity in B-cells has been examined only in I29B, as we and others (43) have found that other cell lines representative of this stage of lymphoid differentiation, in which constitutive c-rel expression is highest (20), are refractory to transfection. Although it is not possible to dismiss the suggestion that the I29B cell line is unusual with respect to c-rel regulation, this seems unlikely as a survey of endogenous c-rel expression in a number of murine B-cell lymphomas has shown that the pattern and level of c-rel mRNA and protein expression in I29B are typical of these cell lines5 (20).

When the octamer sequence upstream of kB1 was shown to contribute to the activity of the c-rel promoter in I29B, it was anticipated that this would be an effect of the B-cell-specific Oct2 protein. However, gel mobility shift assays have demonstrated that this sequence binds Oct1 and Oct2 quite poorly. Binding of Oct2 to octamer sequences in immunoglobulin promoters is associated with a T nucleotide flanking the octamer motif (TNATTTGCAT). A mutation changing this T to an A (as in the c-rel promoter) reduces lymphoid-specific activity to only 10–20% of wild-type (30). Although the Oct1 and Oct2 proteins bind the c-rel octamer sequence only weakly, it cannot be ruled out that the loss of CAT activity seen with mutation is due to the loss of a small but functionally significant level of octamer protein binding.

Binding of complexes from I29B extracts which appear to contain proteins other than Oct1 or Oct2 is dependent on at least part of the c-rel octamer sequence. Although no octamer sequence is found in the chicken c-rel promoter (23, 24), a 24-bp region of the murine promoter (nts −449 to −422, Fig. 1), which includes the octamer sequence, shows significant homology (15/21 identity) to the chicken c-rel promoter (nts −916 to −896; Ref. 24). A sequence in the interleukin 8 promoter thought to bind the transcription factor NF-IL6 (44, 45) has significant homology to both murine and chicken c-rel promoters (8/8 and 6/8 nucleotides, respectively) overlapping with the octamer site. The function of these sequences in the context of the c-rel promoter and Oct, remains to be determined.

Although each of the NF-kB sites in the c-rel promoter contributes to constitutive transcription in mature B-cells, the regulatory role of the different nuclear complexes in I29B that bind to these elements remains to be determined. The kB2 site, although conforming to the consensus NF-kB motif (34), does not bind p50/p65 or p50 homodimers and binds a complex that may be a novel combination of rel family members or unrelated proteins. The p50/p65 complex, a well established activator of transcription, binds to the kB1 and kB3 sites with different affinities, whereas p50 binds to both of these elements with similar affinities. Consistent

5 S. Gerondakis, unpublished results.
with the apparent role of p50/p65 in constitutive c-rel expression in I29B cells is the finding that mature B-lymphocytes are one of the few cell types in which this protein complex is always present in the nucleus (12). The p50 subunit lacks a carboxyl-terminal trans-activation domain (11), and it has been suggested that (p50)2 could act as a negative regulator by occupying sites without activating transcription. This view is supported by cotransfection studies with p50 expression vectors (46). Recent in vitro transcription studies, however, indicated that (p50)2 can adopt different configurations that depend on the sequence to which it is bound and in some conformations can activate transcription (47). Furthermore, the recent finding that the p50 subunit can interact with NF-IL6 (48) raises the possibility that (p50)2 contributes to c-rel transcription in B-cells by interacting with transcription factors that bind sequences adjacent to kB1 and kB3. Consistent with this model is the observation that factor(s) binding to Oct, occupy this site simultaneously with (p50)2 binding to kB1. It remains to be determined which conformation p50 homodimers adopt when bound to these sites and whether positive regulation through kB1 and kB3 is due solely or in part to binding by the p50/p65 complex. The identification of two functional NF-xB sites in the murine c-rel promoter raises the possibility that the diverse patterns of c-rel transcription induced by a variety of stimuli in different cell types (21, 22) could in part be controlled by the regulated recruitment of cytoplasmic rel/NF-xB complexes activated by these signals.

Cotransfection studies have demonstrated that both p75c-rel and a c-rel protein (c-rel CA) lacking the carboxyl-terminal trans-activation domain (amino acids 403–588) are able to up-regulate the c-rel promoter in the I29B cell line. Although c-rel homodimers are able to bind to kB3 in vitro (Fig. 6D), and mutagenesis of the three NF-xB sites in the murine promoter clearly establishes the importance of these elements in the control of constitutive c-rel expression in B-cells, the c-rel mediated up-regulation does not occur through these sites. This finding is reminiscent of studies on the chicken c-rel promoter in which the presence of a NF-xB site located within the basal promoter is not required for repression by v-rel or c-rel (24). Furthermore, in both the chicken and murine promoters, rel proteins appear to exert these effects through the basal promoter region.

The ability of a c-rel protein lacking the potent carboxyl-terminal trans-activating domain to up-regulate c-rel promoter activity as effectively as the wild-type molecule is unexpected. The rel homology domain of p50 has been shown to activate transcription in vitro when bound to certain NF-xB sites. Although this conserved region is retained in the carboxyl-terminal-truncated c-rel protein, trans-activation is independent of the three NF-xB sites in the promoter. Therefore, direct regulation by a mechanism akin to p50 would require the c-rel proteins to bind to a cryptic NF-xB site within the SstI/PstI fragment, like the novel p65/p85c-rel complex that binds to a nonconventional NF-xB site within the human urokinase promoter (16). This model, however, appears unlikely due to the inability of c-rel protein to bind to any sites other than kB3 in the SstI/PstI fragment and the absence of detectable c-rel protein in any nuclear complex isolated from c-rel-transfected cells that bound to sequences within this region of the promoter. Although it is not possible to rule out that c-rel-mediated trans-activation could result from protein-protein interactions that may be disrupted during the preparation of nuclear extracts, this is unlikely, as the inability of the c-rel protein mutant for DNA binding (c-rel ND) to trans-activate the c-rel promoter points to a requirement for DNA binding by c-rel proteins in the trans-activation process. Instead, a model in which c-rel proteins indirectly up-regulate the expression of the c-rel promoter by modulating the expression of other transcription factors, which in turn effect transcription of the c-rel gene, appears to be the favored model. Preliminary studies have revealed no obvious changes in the abundance of nuclear complexes from c-rel-transfected I29B or COS cells binding within the SstI/PstI fragment. However, a more detailed analysis may indicate subtle changes in the composition of these complexes.

The finding that carboxyl-terminal-truncated c-rel is able to up-regulate the c-rel promoter has implications for the mechanism by which v-rel is proposed to transform cells. p59v-rel is thought to be a potent repressor of genes under the control of NF-xB elements due to the loss of the carboxyl-terminal trans-activation domain, thereby creating a rel protein unable to activate transcription but which could disrupt the balance of the rel/NF-xB complexes or compete with endogenous factors at the level of DNA binding. This has led to the proposal that v-rel-mediated transformation may be linked to the down-regulation of certain genes crucial to the control of B-cell growth. This model may, however, be an oversimplification. p59v-rel was originally shown to be a positive regulator of certain promoters (49), whereas c-rel, a known activator, represses the chicken c-rel promoter in embryonic fibroblasts (23, 24). Here, a c-rel protein lacking the carboxyl-terminal trans-activation domain positively regulates the murine c-rel promoter, whereas in T-cells, a c-rel protein lacking the carboxyl-terminal trans-activation domain has also recently been shown to up-regulate interleukin 2 a receptor chain promoter expression as effectively as the wild-type c-rel protein (50). Collectively, these results indicate that rel proteins are able to regulate gene expression in both a positive and negative manner by mechanisms dependent and independent of the carboxyl-terminal trans-activation domain. These activities could be due to the presence of a dominant activator in certain cell types that may override the repressive effect of rel proteins or modification of rel proteins in different cell types such that they operate as activators or repressors. Although carboxyl-terminal truncation is important in the oncogenic activation of c-rel, other lesions in the conserved amino-terminal domain also contribute to the transforming capacity of v-rel (51) and therefore may play a role in the ability of v-rel to repress or activate the expression of particular genes.

Materials and Methods

Cloning and Sequencing of the c-rel Promoter. A murine genomic DNA library generated from Sau3A partial BALB/c liver DNA cloned in EMBL3 was screened by DNA hybridization with a 32P-labeled 0.4-kb EcoRI/NcoI DNA fragment isolated from the 5’ untranslated region of the murine c-rel cDNA, λmc-rel1.2 (6). Three distinct clones were isolated that contained sequences homologous to the probe, and one clone, λmc-rel11, was chosen for more detailed analysis. The sequence of both complementary DNA strands of a 1.4-kb Ncol restriction fragment lying directly upstream of the initiation codon for the c-rel protein was obtained by the dideoxy nucleotide chain termination reaction (52).
RNase Protection and Primer Extension Mapping. For primer extension analysis, a uniformly labeled single-stranded primer corresponding to a 75-bp Smal/Nhel fragment located within the 1.4-kb NcoI fragment was synthesized from an M13 template as described (53). An excess of primer (5 ng) was hybridized to 10 μg of I29B polyadenylated RNA for 2 h in 50 mM formamide-0.4 mM NaCl-1 mM EDTA-0.04 M piperazine-N,N ’-bis(2-ethanesulfonic acid) (pH 6.5), after which the RNA was separated from unannealed primer by oligo-dT cellulose chromatography and reverse transcribed (54). The products were then fractionated on a 5% acrylamide-8 M urea gel.

For RNase protection analysis, 20-μg samples of total cellular RNA isolated from the murine B-cell lymphoma I29B, NIH 3T3 fibroblasts treated with FCS (Flow Laboratories, McLean, VA) plus cycloheximide, or splenic B-cells stimulated with LPS (GIBCO, Grand Island, NY) for the indicated times were hybridized at 50°C (55) to an [α-32P]UTP antisense RNA probe (Brescatel, Adelaide, Australia) that encompassed a 379-bp SstI/Nhel fragment within the 1.4-kb genomic region. After hybridization, samples were diluted in 350 μl of RNase digestion buffer (0.3 M NaCl-10 mM Tris-Cl, pH 7.5-5 mM EDTA containing 8 and 0.4 μg/ml of RNase A (Sigma, St. Louis, MO) and RNase T1 (Sigma), respectively, and incubated at 37°C for 30 min, and the products were precipitated with ethanol before fractionation on a 5% acrylamide-8 M urea gel. All size estimates were made by comparing the mobility of the labeled products with 32P-labeled Haelll-cleaved ßX174 DNA.

Plasmid Constructs. Plasmids HP-cat, RP-cat, TP-cat, and SP-cat consisted of the 1154-bp HindIII/PstI, 915-bp Rsal/PstI, 672-bp TaqI/PstI, and 476-bp SstI/PstI fragments, respectively, from the 1.4-kb NcoI c-rel genomic fragment inserted upstream of the CAT gene in the promoterless reporter plasmid pCAT3 (31). For all other c-rel promoter CAT constructs, a modified version of HP-cat (Psd6m-HP-cat) was used in which the nucleotide at position -476 (Fig. 1) was changed from C to G to generate a Psdi site encompassing residues -473 to -478. Octi6m-HP-cat, kb1m-HP-cat, kb2m-HP-cat, and kb3m-HP-cat are derivatives of the Pstdim-HP-cat plasmids with either the oct or one of each of the three NF-κB motifs within the 1.4-kb NcoI c-rel genomic fragment altered by in vitro mutagenesis. The plasmids octi6m-HP-cat, octm4-HP-cat, and octm3:kb3m-HP-cat represent Pstdim-HP-cat with the oct and one of each of the κB motifs altered by in vitro mutagenesis, respectively, whereas kb1m2p3m-HP-cat represents Pstdim-HP-cat with all three of the κB sites mutated simultaneously. A diagram of all c-rel promoter constructs is presented in Fig. 3A.

The c-rel expression vector, pDAMP56 c-rel, consisted of a 2229-bp Nhel/HindIII cDNA fragment encompassing the entire coding region of the murine c-rel protein cloned into the eukaryotic expression vector pDAMP56, whereas the expression plasmid pcDNA c-rel/CAT represents the 1208-bp Ncol/Scal fragment of the murine c-rel cDNA (amino acids 1-403), which lacks the c-rel trans-activation domain (9), and inserted in pcDNA1 (Invitrogen, San Diego, CA). The expression plasmid pcDNA1c-reLNΔ contains a modified Nhel/HindIII cDNA fragment in which sequences corresponding to amino acids 21-135 have been deleted. The integrity of all inserts was verified by restriction mapping, and, in the case of all clones derived by in vitro mutagenesis, by sequencing. All plasmids used for transfection were purified twice with cesium chloride equilibrium gradient centrifugation.

Cell Culture. A clonal variant of the murine B-cell lymphoma I29 (I29B) was maintained in RPMI 1640 medium containing 8% heat-inactivated FCS and 50 μM 2-mercaptoethanol, and the human T-cell line Jurkat was maintained in human tonicity RPMI 1640 supplemented with 7% FCS. NIH 3T3 fibroblasts were maintained in DME supplemented with 10% FCS, whereas monkey COS cells were grown in DME supplemented with 5% FCS. For inductions of c-rel mRNA, confluent short-term passage NIH 3T3 fibroblasts were serum starved in DME containing 0.25% FCS for 24 h prior to stimulation for 2 h with 20% FCS and cycloheximide at a concentration of 10 μg/ml, conditions shown previously to induce maximal levels of c-rel mRNA (22). B-cells were isolated from the spleens of 6-8-week-old BALB/c mice, and T-cells were eliminated from these preparations using antibody-complement-mediated lysis. Viable B-cells were culled at a density of 5 × 10⁶ cells/ml in RPMI 1640 medium containing 10% FCS, 50 μM 2-mercaptoethanol, and 25 μg/ml of LPS.

Transfections. For all transient transfections with c-rel CAT reporter gene plasmids, approximately 2 × 10⁶ I29B or 5 × 10⁶ Jurkat cells were transfected by a modification of the DEAE-dextran method (56), and transient COS cell transfections with the Oct2-containing expression vectors pOEV1 + and pOEV1 - (57) were performed by the DEAE-dextran method (58). When indicated, 4 h after transfection, Jurkat cells were stimulated with 40 ng/ml PMA (Sigma). For the Jurkat and I29B transfections, approximately 3 and 10 μg, respectively, of each promoter-CAT reporter construct were used. The precise amount of each plasmid was varied slightly to ensure transfection of an equivalent molar amount of each DNA sample. For the I29B transfections, a 3-fold molar excess of the rel expression vector DNA (30-35 μg, depending on the rel insert) was used. Approximately 48 h after all transfections, cells were washed in mouse toxicity phosphate-buffered saline and resuspended in 0.1 ml of 0.25 M Tris-Cl, pH 7.5, and extracts were prepared by several rounds of freeze/thawing, followed by centrifugation. CAT activity was determined from lysate samples that had been equalized for protein content (25 μg of total protein/transfection) essentially as described (59), and after thin-layer chromatography followed by autoradiography, CAT activity was quantitated using phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). CAT activity in each transfection is expressed as the percentage of acetylated 14C-labeled chloramphenicol as compared with the total [14C]chloramphenicol. All results presented represent a mean of at least five separate transfections, using, in each case, two separate plasmid preparations. Approximately 10-20% variance was observed among replicate experiments. In some cases, results were confirmed by cotransfection with a plasmid containing the luciferase gene coupled to the Rous sarcoma virus promoter (pRSV1) (60). Luciferase activity was assayed according to the manufacturer’s specifications (Promega) and measured in a luminometer (Berthold).

Synthesis of c-rel Protein in E. coli. The rel homology domain of p75c-rel was expressed in E. coli as a fusion with the 28 kilodalton Spirillum japonicum GST protein in the vector pGex-2T (61). The DNA binding domain of the murine c-rel protein encompassed by a 1.2-kb NcoI/Scal fragment (amino acids 1-403) was inserted into a derivative of pGex-2T and transformed into the E. coli strain JPA101, with
the induced expression and purification of all soluble GST fusion proteins using glutathione S-agarose (Sigma), performed essentially as described (61).

**Electrophoretic Mobility Shift Assay.** Nuclear extracts were made as described (62) with the following modifications. Briefly, 10^6 to 10^7 cells were harvested, washed in mouse tonicity phosphate-buffered saline, suspended in 500 µl of buffer A (10 mM Hepes, pH 7.9–10 mM KCl-0.1 mM EDTA-0.1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate acid-1 mM dithiothreitol-0.5 mM phenylmethylsulfonyl fluoride-4 µg/µl leupeptin, and then pelleted. Cells were gently resuspended in 400 µl buffer A and allowed to swell on ice for 15 min. After the addition of 25 µl of 10% Nonidet P-40, samples were vortexed for 10 s and centrifuged at 12,000 × g for 30 s. Then 50 µl of buffer C (420 mM NaCl-20 mM Hepes, pH 7.9–1.5 mM MgCl-0.2 mM EDTA-25% glycerol) were added to the pellet, and the tube was agitated every few minutes for a period of 20 min. The extract was centrifuged for 5 min at 10,000 × g, and the supernatant was frozen at −70°C. Extracts typically contained 0.5–2 µg of protein/µl.

For probes, oligonucleotides or appropriate restriction fragments were cloned into the SP72 vector, digested with restriction enzymes, and end labeled using the Klenow fragment of E. coli DNA polymerase 1 and [α-32P]dATP. Oct, and Octα, are the 88-bp PstI-Smal fragment (nts −476 to −388; Fig. 1) containing the Kb1 promoter, mutation or Kb1, and Octα, respectively. Oligonucleotides (Applied Biosystems 381A synthesizer) were: Kb1: 5'-GGGGGACTTCTCCGAGACG3'- (33)Kb1: 5'-TGGGAGAGGGGAAAACCTTG AAAA-3'Kb2: 5'-TGGGAGAGGGGAAAACCTT GGACGACGCT-3'Kb3: 5'-GCTAAGCAGGGGAAATTC CCCCCTTCCTCC-3'Octα: 5'-TAAATTTGTACT-3' (63)

Electrophoretic mobility shift assay reactions contained 5000 cpm of 32P-labeled probe, 0.1–1 µg poly[dI-dC], 0.25–4 µg protein, and reaction buffer (10 mM Tris, pH 7.5–100 mM NaCl-1 mM EDTA-5% glycerol-0.1% Nonidet P-40 for binding to NF-kB sites; 20 mM Hepes-40 mM KCl-0.2 mM EDTA-1.2 mM MgCl-5% glycerol for binding to Oct sites) in a total volume of 15 µl. For competition experiments, competitor DNA was incubated with extract for 10–15 min on ice before addition of probe. When antibodies specific for Oct1 (39), Oct2 (40), p50 (38), and murine c-rel (Santa Cruz Biotechnology, Santa Cruz, CA) proteins were used in gel shift assays, extract and poly[dI-dC] were incubated on ice for 10 min, antibody was then added, and incubation on ice continued for 10 min before addition of probe. The incubation was continued for 15–20 min at room temperature, and then 2 µl of dye containing Ficoll were added, after which reactions were loaded on 5% nondenaturing polyacrylamide gels and run at 150 V for 35 min. After drying, gels were exposed overnight to Kodak XAR5 films at −70°C with an intensifying screen.

**PCR-mediated Mutagenesis.** PCR-mediated mutagenesis was performed essentially as described by Ho et al. (64) using standard PCR protocols except for the mutagenesis of kb2 and kb3 (see below). In all cases, the outer primers corresponded to regions in the T7 and SP6 promoters; the inner oligonucleotides containing the mutations are shown in Table 1. To allow the isolation of the oct sequence on a convenient restriction fragment, a single base change (C to G) at position −476 was made, creating a PstI site. All constructs thereafter contained this change. Mutations were created using the indicated oligonucleotides with either the T7 or SP6 primers to produce fragments varying in length from 50 to 450 bp. These PCR products were purified from low-melt agarose gels, and the pairs were mixed and used in reactions with T7 and SP6 primers to produce a full-length product containing the mutation. These were again purified from low-melt agarose gels, digested with appropriate restriction enzymes, recoloned, and sequenced to verify the presence of mutations. Mutated clones were generated by replacing the wild-type HindIII-Pst fragment of the murine c-rel promoter (see Fig. 3, nts −880 to +273) with one of several mutated fragments.

Owing to the high GC content of the region containing kb2 and kb3, a normal PCR protocol failed to give a product when clones containing regions from −389 bp to +104 bp of the c-rel promoter were used as template. In order to overcome this problem, it was necessary to use 7-deaza-dGTP (c7dGTP) in the reaction at a ratio of 50 (c7dGTP):50 (dGTP) and to increase the number of cycles from 30 to 40.

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