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Inhibition of T-cell proliferation by a MYB antisense oligomer is accompanied by selective down-regulation of DNA polymerase α expression

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ABSTRACT **We recently found that inhibition of MYB protein synthesis in human peripheral blood mononuclear cells (PBMC) exposed to human c-*myb* (designated *MYB*) antisense oligodeoxynucleotides prevents entry into S phase and cell proliferation. To determine the mechanism(s) by which down-regulation of human c-myb protein (MYB) synthesis interferes with DNA synthesis, we analyzed mRNA levels of DNA polymerase α and proliferating cell nuclear antigen (PCNA), transcripts of two genes required for DNA synthesis, in normal and leukemic T lymphocytes exposed to *MYB* antisense oligodeoxynucleotides. Expression of DNA polymerase α was inhibited both in normal T lymphocytes progressing from G_0 to S phase and in exponentially growing CCRF-CEM leukemic cells, whereas expression of PCNA was inhibited only in mitogen-stimulated PBMC and remained essentially unaffected in the leukemia T-cell line. The functional link between expression of MYB and DNA polymerase α mRNAs was further demonstrated by analyzing DNA polymerase α mRNA levels in a temperature-sensitive (ts) fibroblast cell line (TK$^-$ts13; TK is thymidine kinase) constitutively expressing human MYB mRNA driven by the simian virus 40 (SV40) promoter. In the MYB-expressing TK$^-$ts13 cells, DNA polymerase α mRNA levels were unaffected following shift to the nonpermissive temperature of 39.6°C, whereas in the parental line, DNA polymerase α mRNA levels were readily down-regulated. These findings indicate that the expression of MYB is related to that of DNA polymerase α in cells expressing MYB at high levels and suggest that there is a functional link between c-myb and DNA polymerase α mRNA expression during cell cycle progression of normal T lymphocytes.**

The protooncogene c-*myb* has long been implicated as a regulator of T-lymphocyte proliferation. Until recently, the evidence was only indirect and based on the observation that the transcription of c-*myb* and steady-state mRNA and protein levels increase in response to phytohemagglutinin (PHA) and interleukin 2 and peak in S phase (1–5); the finding that c-myb mRNA levels fluctuate in cycling lymphoid cells, with highest expression during S phase (6), further emphasized the importance of this gene in T-lymphocyte proliferation.

We showed previously that, in human peripheral blood mononuclear cells (PBMC) cultured with PHA in the presence of a human c-*myb* (designated *MYB*) antisense oligomer, c-myb protein (MYB) synthesis and lymphocyte proliferation are substantially inhibited (7). Inhibition of MYB protein synthesis did not affect early events in T-lymphocyte activation such as expression of the interleukin 2 receptor and MYC but appeared to specifically inhibit the G_1/S transition as indicated by down-regulation of the S-phase marker gene encoding histone H3 (8).

Recently, v-myb and c-myb proteins were shown to encode a transactivating domain that up-regulates the expression of reporter genes linked to binding sites of DNA binding domains (9–11). The possibility that c-myb might have a similar role *in vivo* has prompted the search for cellular genes regulated by c-myb. Our previous studies indicated, in both the hematopoietic and lymphoid systems, that the expression and function of c-myb is tightly linked to the cellular entry into S phase and DNA synthesis (7, 12, 13). We therefore focused the present analysis on two genes of the DNA-synthesizing machinery, genes for PCNA and DNA polymerase α (14–17), and the effects of inhibition of MYB protein synthesis on their expression. DNA polymerase α plays a key role in the replication of the eukaryotic genome (18–20), while PCNA gene appears to be needed for *in vitro* simian virus 40 (SV40) DNA replication and cell cycle progression of mammalian cells (21, 22). The steady-state mRNA levels of DNA polymerase α and PCNA increase sharply during the G_0–S transition, whereas in cycling cells, the levels remain essentially constant in different phases of the cell cycle (14, 17). Our studies indicate a tight link between inhibition of MYB protein synthesis and down-regulation of DNA polymerase α expression in normal T lymphocytes progressing from G_0 to S phase and in exponentially growing leukemic T lymphocytes; in contrast, PCNA expression is down-regulated in normal T lymphocytes and unaffected in the leukemic cell line.

MATERIALS AND METHODS

Cells and Culture Conditions. PBMC were isolated from 50 ml of venous blood from a healthy donor. Upon isolation, the cells were comprised of 70% T lymphocytes, 10% B lymphocytes, and 20% monocytes as determined by flow cytometric analysis. Cells were cultured at 37°C in a humidified 5% CO_2/95% air atmosphere at 5×10^5 cells per ml in 24-well microtiter plates (Costar) in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum, antibiotics, and 5 μg of PHA per ml. After 30 hr of incubation, some wells were supplemented with either *MYB* sense (14 μM) or antisense (14 μM) oligodeoxynucleotides corresponding to or complementary to codons 2–7 of human MYB mRNA (7, 23). CCRF-CEM cells (24) (a cell line established from a child with T-cell leukemia) were grown in suspension culture in RPMI 1640 medium containing 10% fetal bovine serum at 1×10^6 per ml in 24-well microtiter plates in the presence of 14 μM of either *MYB* sense or antisense oligodeoxynucleotides.

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Abbreviations: PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells; ts, temperature sensitive; TK, thymidine kinase; PCR, polymerase chain reaction; RT, reverse transcriptase; hnRNA, heterogeneous nuclear RNA; SV40, simian virus 40.

Cells were harvested at various time points, and RNA was extracted as described (25).

RNA Phenotyping in Normal and Leukemic T Lymphocytes by Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis. mRNA levels of MYB, PCNA, DNA polymerase α, and β_2-microglobulin in normal and malignant T lymphocytes were analyzed as follows. Cells seeded at 1×10^6 per ml were collected at the end of the culture period, and total RNA was extracted in the presence of 20 μg of *Escherichia coli* ribosomal RNA as described (25). RNA was then reverse-transcribed by using 400 units of Moloney murine leukemia virus RT and 0.2 μg of oligo(dT) as primer for 1 hr at 37°C. The resulting cDNA fragments were amplified with 5 units of *Thermus aquaticus (Taq)* polymerase as described (26) in the presence of synthetic primers specific for each mRNA. Each specific primer was selected according to the published nucleotide sequence of human MYB (23), PCNA (14), DNA polymerase α (16), and β_2-microglobulin cDNAs (27). Table 1 lists the location of each primer in the corresponding cDNA sequence. Twenty microliters of the 100-μl PCR reaction was separated in a 4% NuSieve agarose gel and transferred to a nitrocellulose filter. The resulting blots were hybridized with synthetic 50-base oligodeoxynucleotide probes complementary to the amplified mRNA. The synthetic oligomers were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase as described (28).

To measure the steady-state levels of PCNA mRNA precursor [PCNA heterogeneous nuclear RNA (hnRNA)] in oligodeoxynucleotide-treated cultures, we utilized the technique recently described by Lipson and Baserga (29) to detect the thymidine kinase (TK) pre-mRNA in human fibroblasts (WI-38 cells). The 5′ PCNA primer corresponds to nucleotides 244–265 in the first exon, and the 3′ primer corresponds to nucleotides 574–595 in the first intron. The amplification products were transferred to a nitrocellulose filter and hybridized to an end-labeled synthetic oligomer corresponding to nucleotides 457–490 in intron 1. The nucleotide designation is based on the published sequence of the human PCNA gene (30).

PCNA and DNA Polymerase α mRNA Stability in CCRF-CEM Cells. The half-life of PCNA and DNA polymerase α mRNAs was determined in 2×10^7 CCRF-CEM cells exposed to 2.5 μg of actinomycin D per ml for 0, 1, 2, 4, and 8 hr; cells were collected, and RNA was extracted as described (31). mRNA stability was measured by RNA blot-hybridization (Northern) analysis of actinomycin D-treated RNA with PCNA and DNA polymerase α cDNA inserts ^{32}P-labeled by the random priming method (32).

DNA Polymerase α mRNA Levels in MYB-Expressing TK$^-$ts13 Cells at the Restrictive Temperature of 39.6°C. DNA polymerase α mRNA levels were determined in the temperature sensitive (ts) parental cell line TK$^-$ts13 and in a derivative cell line, SVmybTK$^-$ts13 containing human TK cDNA and human MYB cDNA driven by the early SV40 promoter, after shifting at the nonpermissive temperature of 39.6°C. Cells were collected at 0, 2, 4, and 8 hr, and RNA was extracted from 1×10^6 cells at each time point; a fifth of the extracted RNA was used for RT-PCR analysis at each point.

Table 1. Synthetic primers used for mRNA phenotyping in normal and leukemic T lymphocytes

Primer	Inclusive nucleotide positions in genes			
	MYB	PCNA	polα	β_2-Microglobulin
5′	2258–2279	244–265	3969–3990	280–301
3′	2466–2487	404–425	4187–4208	510–531

polα, DNA polymerase α.

RESULTS

Expression of MYB mRNA in PHA-Stimulated PBMC Exposed to *MYB* Sense and Antisense Oligodeoxynucleotides. We recently showed that exposure of PBMC to *MYB* antisense oligodeoxynucleotides determines down-regulation of MYB mRNA expression (7). To analyze the kinetics of MYB mRNA expression in PBMC and thereby determine the exposure time required to observe down-regulation of MYB mRNA expression, PBMC were exposed to PHA for 30 hr and then to *MYB* sense or antisense oligodeoxynucleotides for an additional 8 hr. After this 8-hr period, cells were collected at 0, 4, 8, 12, and 24 hr, RNA was extracted, and expression of MYB was analyzed by the RT-PCR technique.

MYB mRNA was detectable at 8, 12, and 24 hr in the sense-treated culture and at much lower levels in the antisense-treated cultures (Fig. 1). The kinetics of MYB mRNA expression revealed by the RT-PCR technique closely mimics that reported from standard Northern blot analysis (1–3). Based on the decreasing intensity of the bands with time, we conclude that there is specific MYB mRNA degradation in antisense-treated culture at each time point in which MYB mRNA molecules are detected by PCR-RT amplification; in addition, a 12-hr exposure of PBMC to *MYB* antisense oligodeoxynucleotides is apparently sufficient to drastically reduce MYB mRNA levels.

Expression of PCNA and DNA Polymerase α mRNAs. *Expression in PHA-stimulated PBMC exposed to* MYB *sense and antisense oligodeoxynucleotides.* To determine whether inhibition of *MYB* expression affects expression of genes directly involved in DNA synthesis, we studied PCNA and DNA polymerase α mRNA levels in PBMC treated with PHA for 30 hr and subsequently exposed to *MYB* sense and antisense oligodeoxynucleotides. Inhibition of *MYB* gene function by exposure to antisense oligodeoxynucleotides was accompanied by a significant decrease in the accumulation of PCNA mRNA detected in *MYB* sense-treated cultures (Fig. 2) and in the culture treated with PHA alone (not shown). Similarly, DNA polymerase α expression was also decreased when *MYB* expression was inhibited; by contrast, expression of β_2-microglobulin gene was essentially unaffected (Fig. 3). These results suggest that c-myb mRNA down-regulation is accompanied by a specific effect on PCNA and DNA polymerase α mRNA levels.

Expression in exponentially growing leukemic cells (CCRF-CEM) exposed to MYB *sense and antisense oligode-*

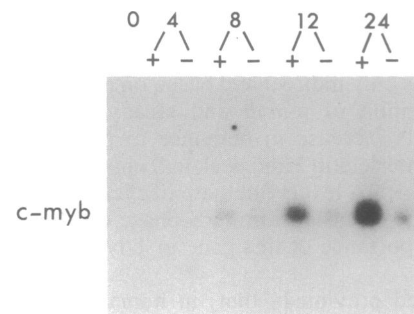

FIG. 1. Kinetics of MYB mRNA expression in PHA-stimulated PBMC in the presence of *MYB* sense or antisense oligodeoxynucleotides. Total RNA was isolated from PBMC and reverse-transcribed, amplified, and hybridized to a synthetic 30-base MYB cDNA fragment localized in the amplified sequence as described (7). Numbers indicate the times (hr) at which PBMC were collected and RNA was extracted after PHA stimulation for 30 hr; exposure to *MYB* sense (lanes +) or antisense (lanes −) oligonucleotides was for an additional 8 hr. After the 8-hr incubation with *MYB* oligodeoxynucleotides, the medium was not changed, so that total exposure time to the oligomers was 8 hr plus 4, 8, 12, or 24 hr.

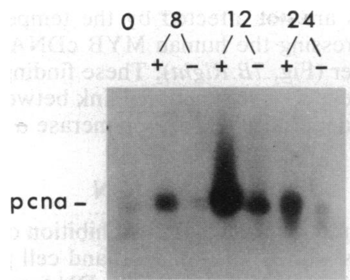

FIG. 2. Expression of PCNA mRNA in PBMC stimulated with PHA in the presence of *MYB* sense or antisense oligodeoxynucleotides. The experimental conditions were as described in the legend to Fig. 1 except that PCNA-specific primers were used for the amplification of PCNA mRNA and a PCNA synthetic fragment was used as the hybridization probe.

oxynucleotides. To determine whether *MYB* inhibition also coincided with decreased expression of PCNA and DNA polymerase α mRNAs in exponentially growing cells, CCRF-CEM leukemia cells were exposed to *MYB* sense and antisense oligodeoxynucleotides, and mRNA levels of c-myb, PCNA, and DNA polymerase α were analyzed. In CCRF-CEM cells exposed to *MYB* antisense oligodeoxynucleotides for 12–24 hr, MYB mRNA levels were down-regulated, whereas these levels were unaffected during the initial 8 hr of exposure (Fig. 4). DNA polymerase α mRNA levels were also decreased at 12 and 24 hr (Fig. 4). By contrast, the expression of PCNA mRNA remained relatively constant in *MYB* sense- and antisense-treated cells from 0 to 24 hr (Fig. 4).

Half-life of PCNA and DNA polymerase α mRNAs in CCRF-CEM cells. To determine whether the differential effect on the expression of PCNA and DNA polymerase α mRNAs in exponentially growing CCRF-CEM cells was due in part to different stabilities of PCNA and DNA polymerase α mRNAs, the half-life of these mRNAs was measured.

Exponentially growing CCRF-CEM cells were exposed to 2.5 μg of actinomycin D per ml, and total RNA was isolated at time 0 and at 1, 2, 4, and 8 hr after addition of actinomycin D. The hybridization signal obtained with the DNA polymerase α cDNA probe is virtually undetectable in CCRF-CEM cells exposed to actinomycin D for 2 hr, indicating that the half-life of DNA polymerase α mRNA is less than 2 hr; in marked contrast, PCNA steady-state mRNA levels were still readily detectable at 8 hr (Fig. 5); the modest decrement in

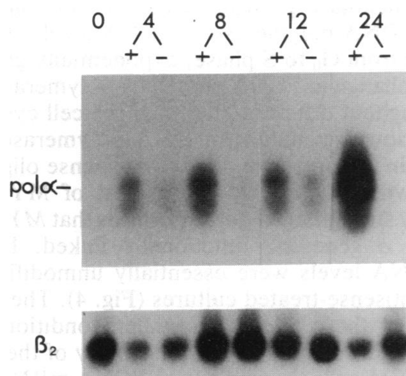

FIG. 3. Expression of DNA polymerase α (polα) and β_2-microglobulin (β_2) mRNAs in PBMC stimulated with PHA in the presence of *MYB* sense or antisense oligodeoxynucleotides. The experimental conditions were as described in the legend to Fig. 1 except that (*i*) DNA polymerase α- and β_2-microglobulin-specific primers were used for amplification of the respective mRNAs and (*ii*) DNA polymerase α and β_2-microglobulin synthetic fragments were used as hybridization probes.

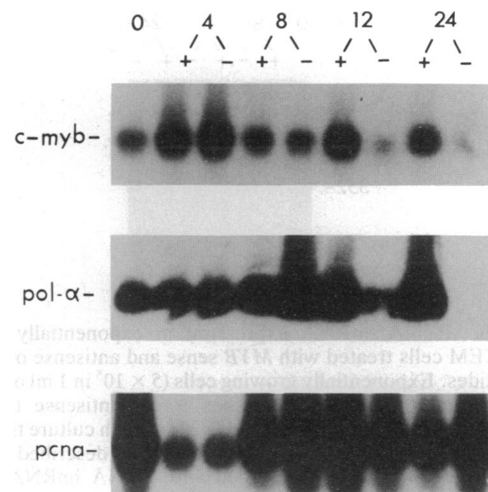

FIG. 4. Expression of MYB, DNA polymerase α (polα), and PCNA mRNAs in exponentially growing CCRF-CEM cells treated with *MYB* sense and antisense oligodeoxynucleotides. Numbers indicate the times (hr) at which CCRF-CEM cells were collected and RNA was extracted after exposure to *MYB* sense (lanes +) or antisense (lanes −) oligodeoxynucleotides.

PCNA mRNA levels at 4 and 8 hr compared with the untreated cultures indicates that the half-life of PCNA mRNA is much longer than that of DNA polymerase α in CCRF-CEM cells.

Expression of PCNA hnRNA in Exponentially Growing Leukemic Cells (CCRF-CEM) Exposed to *MYB* Sense and Antisense Oligodeoxynucleotides. The much longer half-life of PCNA mRNA compared with that of DNA polymerase α (Fig. 6) raises the possibility that inhibition of MYB protein synthesis might still affect PCNA expression at the transcriptional level, and yet the effect was not observed by measuring PCNA steady-state mRNA levels. To test this hypothesis, PCNA steady-state pre-mRNA levels were measured in exponentially growing CCRF-CEM cells exposed to *MYB* sense or antisense oligodeoxynucleotides for as long as 24 hr.

A 370-base-pair (bp) segment extending from the first exon into the first intron of PCNA hnRNA was amplified by the RT-PCR technique and detected by hybridization with an intron 1-specific probe. The steady-state hnRNA levels of PCNA were essentially unaffected in the *MYB* antisense-treated cultures in comparison with the *MYB* sense-treated cultures (Fig. 6). These findings indicate that the inhibition of MYB protein synthesis does not affect PCNA expression in exponentially growing leukemia cells.

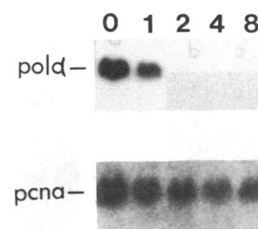

FIG. 5. Half-life of DNA polymerase α (polα) and PCNA mRNAs in exponentially growing CCRF-CEM cells. Exponentially growing cells (25×10^7 in 50 ml of medium) were exposed to actinomycin D at a final concentration of 2.5 μg/ml for 0, 1, 2, 4, and 8 hr; at each time point, cells were collected and total RNA was isolated by the procedure as described (31). Total RNA (15 μg) for each time point was electrophoresed in 1.2% agarose/formaldehyde gel and transferred to nitrocellulose as described by Thomas (33). Hybridization was carried out first with a DNA polymerase α cDNA (16) and subsequently with a PCNA cDNA (15) as described (34).

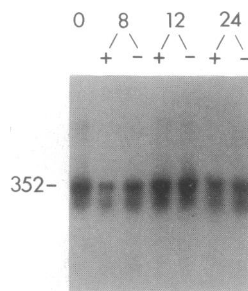

FIG. 6. PCNA hnRNA expression in exponentially growing CCRF-CEM cells treated with *MYB* sense and antisense oligodeoxynucleotides. Exponentially growing cells (5×10^5 in 1 ml of culture) were exposed to *MYB* sense (lanes +) or antisense (lanes −) oligodeoxynucleotides for 8, 12, and 24 hr; at each culture time, cells were collected and total RNA was isolated as described (25, 29). Total RNA was reverse-transcribed with PCNA hnRNA-specific primers, amplified, and hybridized to a synthetic 40-base PCNA intron 1 fragment; 352 indicates the amplified PCNA segment in bp.

DNA Polymerase α mRNA Levels in TK$^-$ts13 Fibroblasts Constitutively Expressing Human MYB mRNA. ts13 cells are derived from Syrian baby hamster kidney (BHK) cells and were originally described by Talavera and Basilico (35). They have a *ts* mutation that stops cell cycle progression in the G_1 phase at the restrictive temperature of 39.6°C. TK$^-$ts13 cells were cotransfected with a human c-myb cDNA driven by the early SV40 promoter (36) and a human TK cDNA (37). TK$^+$ colonies were selected in hypoxanthine/aminopterin/thymidine (HAT) medium; total RNA and proteins were extracted from mixed cell populations and found to contain high levels of MYB mRNA and protein (Fig. 7*A*). We then asked whether MYB would up-regulate the expression of DNA polymerase α mRNA when TK$^-$ts13 cells were shifted at the restrictive temperature of 39.6°C. Since the half-life of DNA polymerase α mRNA is relatively short, we would expect an early decline of DNA polymerase α mRNA in the parental TK$^-$ts13 cells; in contrast, DNA polymerase mRNA levels should not be down-regulated in TK$^-$ts13 cells expressing high levels of MYB mRNA. Cells exponentially growing at the permissive temperature were placed in 1% fetal bovine serum for 12 hr and then shifted to the restrictive temperature of 39.6°C; cells were collected for RNA extraction 0, 2, 4, and 8 hr later; DNA polymerase α expression was analyzed in the parental cell line and in the cell line containing the human MYB cDNA driven by the SV40 promoter by RT-PCR analysis. It is rather obvious that the expression of DNA polymerase mRNA is readily down-regulated in TK$^-$ts13 parental cells and is undetectable 8 hr after the temperature shift (Fig. 7*B Left*), while DNA polymerase α mRNA levels are not affected by the temperature shift in TK$^-$ts13 expressing the human MYB cDNA driven by the SV40 promoter (Fig. 7*B Right*). These findings further indicate that there is a close functional link between the expression of *MYB* and that of DNA polymerase α gene.

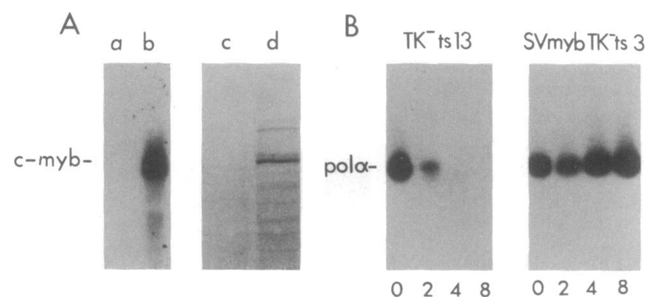

FIG. 7. Expression of myb and DNA polymerase α (polα) mRNAs in TK$^-$ts13 cells and human MYB-expressing TK$^-$ts13 cells at the restrictive temperature of 39.6°C. (*A*) MYB mRNA and protein levels were determined by Northern and Western blot techniques in TK$^-$ts13 cells (lanes a and b) and in SVmybTK$^-$ts13 cells (lanes c and d). (*B*) DNA polymerase α mRNA levels were determined by RT-PCR technique as described in the legends to Figs. 1–4.

DISCUSSION

The recent demonstration that the inhibition of *MYB* expression interferes with G_1/S transition and cell proliferation in PHA-stimulated PBMC (7) and with DNA synthesis in murine cytolytic T-lymphocyte clones (38) underlines the importance of investigating the molecular mechanisms utilized by MYB to regulate T-lymphocyte proliferation.

Recently, it has been shown that MYB protein, in addition to the DNA-binding domain, encodes a trans-activating domain. It appeared reasonable to ask whether MYB is involved in the regulation of genes of the DNA-synthesizing machinery that are coordinately expressed at the onset of S phase.

In the present studies we investigated the relationship between inhibition of *MYB* function and the expression of PCNA and DNA polymerase α genes. We showed previously that PBMC preincubated with *MYB* antisense oligodeoxynucleotides before PHA stimulation and supplemented with equal amounts of antisense oligodeoxynucleotides at 0, 24, and 48 hr after PHA stimulation do not synthesize DNA and do not proliferate (7). Under these conditions, the expression of DNA polymerase α is completely abrogated (not shown). In the present experiments we added *MYB* sense and antisense oligodeoxynucleotides to PBMC cultures 28–30 hr after PHA addition; we also determined that down-regulation of MYB mRNA expression in antisense-treated cultures becomes evident at 12 hr after treatment. Thus, our experiments were designed to inhibit MYB protein synthesis in late G_1, 36–40 hr after PHA stimulation, shortly before the entry into S phase, which normally begins 48 hr after PHA stimulation. Under these conditions, we detected inhibition of DNA synthesis and T-cell proliferation to the same extent as that observed in PBMC preincubated with *MYB* antisense oligodeoxynucleotides and supplemented with them at 0, 24, and 48 hr after PHA addition. Equally important, in this experimental setting, is the down-regulation of PCNA and DNA polymerase α gene expression at each time point in *MYB* antisense-treated cultures compared with *MYB* sense-treated cultures.

We next asked whether inhibition of MYB protein synthesis in exponentially growing leukemic cells also affected PCNA and DNA polymerase α mRNA levels. Unlike cells progressing from G_0 to S phase, exponentially growing cells show little change in PCNA and DNA polymerase α mRNA levels throughout different phases of the cell cycle (17, 39). Thus, the down-regulation of DNA polymerase α mRNA expression in the presence of *MYB* antisense oligodeoxynucleotides, with kinetics similar to that of MYB (Fig. 4), significantly strengthened our hypothesis that *MYB* and DNA polymerase α gene are functionally linked. In contrast, PCNA mRNA levels were essentially unmodified in *MYB* sense- or antisense-treated cultures (Fig. 4). The differential expression of these two genes under conditions of MYB inhibition might reflect a different stability of their mRNAs; in CCRF-CEM cells the half-life of PCNA mRNA appeared to be much longer than that of DNA polymerase α mRNA (Fig. 5). Alternatively, it is possible that *MYB* acts directly to regulate expression of DNA polymerase α gene, whereas the effect on PCNA gene, as observed during G_0–S transition in PHA-stimulated PBMC, is only indirect. To further address this question, we measured the steady-state levels of PCNA nuclear pre-mRNA in CCRF-CEM cells exposed to *MYB* sense or antisense oligodeoxynucleotides for 8, 12, and 24 hr.

Our results indicate that the transcription of PCNA gene from the first to the second exon is essentially unaffected by inhibition of MYB protein synthesis (Fig. 6). Finally, constitutive expression of a human MYB cDNA in a ts fibroblast line maintains DNA polymerase α gene expression at the nonpermissive temperature, whereas DNA polymerase α mRNA levels are readily down-regulated in the parental line not expressing MYB mRNA at detectable levels (Fig. 7).

Taken together, our results indicate that there is a functional link between the expression of a nuclear protooncogene and a key factor for DNA synthesis; specifically, it appears that down-regulation of DNA polymerase α expression and inhibition of MYB protein synthesis are tightly, and perhaps directly, linked in PBMC progressing from G_0 to S phase and exponentially growing CCRF-CEM cells exposed to *MYB* antisense oligodeoxynucleotides. A proof that *MYB* directly regulates DNA polymerase α gene expression awaits the demonstration of a direct interaction of MYB protein with the DNA polymerase α gene promoter. No matter what the results of that analysis will be, our studies establish that there is an interesting functional link between *MYB* and DNA polymerase α gene expression.

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