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Detection of a countertranscript in promyelocytic leukemia cells HL60 during early differentiation by TPA

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We have isolated several cDNA clones corresponding to the mRNAs expressed during early phase of differentiation of promyelocytic leukemia cells HL60 by TPA. Two interrelated clones were examined, one pHH81 and the other pHH58 possesing inserts of 180 and 730 base pairs, respectively. Northern blot analyses of poly(A) RNAs from induced cells revealed that the clone pHH81 hybridized with 4.3kb RNA, while the clone pHH58 hybridized with 4.3kb and, in addition 0.7kb RNA. Sequence determination of those cDNA clones and extensive Northern blot analyses revealed that the inserts of these clones were derived from 4.3kb mRNAs. 0.7kb RNA was hybridized with only 5' upstream region of the clone pHH58, especially with the strand designed to detect anti-sense RNA. Thus we concluded that 0.7kb RNA is a countertranscript of 4.3kb RNA expressed during early differentiation of HL60 cells.

Human promyelocytic leukemia cells HL60 can be induced to redifferentiate into macrophages by 12-0-tetradecanoylphorbol-13-acetate (TPA) (1-6). HL60 cells seemed to be fully commited to redifferentiation by as short as 4 hr contact with TPA, and thereafter almost all population of the cells can be

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differentiated into macrophages without TPA (2). Thus HL60 cells should offer a good system to analyze the expression and the regulation of the genes involved in the early stage of terminal differentiation in vitro. Recently activation of c-fos (7-9) and inactivation of c-myc genes (10,11) have been shown to accompany redifferentiation of HL60 cells, suggesting that some newly synthesized mRNAs play a key role in redifferentiation of HL60 cells by TPA. Thus, it would be of great interest and importance to characterize mRNAs newly synthesized in redifferentiation of HL60 by TPA, and to elucidate regulatory mechanisms operating in the expression of these genes and their products, which lead HL60 cells into macrophages. To achieve this purpose, it would be useful to obtain specific clones corresponding to the mRNAs which would meet the criteria described above, and to analyze the structural and functional relationships between them. In this paper, we described the isolation of several cDNA clones corresponding to the mRNAs expressed early in TPA-induced differentiation of HL60 cells. By using two of the clones we identified a countertranscript by nucleotide sequence determination and Northern blot analyses.

MATERIALS AND METHODS

Cell culture: Human promyelocytic leukemia cells HL60 were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum.

Nucleotide sequence analyses: Nucleotide sequence was determined by the chemical degradation method of Maxam and Gilbert (12). Restriction enzyme fragments were labeled at the 3' end with calf thymus terminal deoxynucleotidyl transferase and [α-^{32}P]dideoxy ATP (500 Ci/mmol, Radiochemical Center, Amersham) or labeled at the 5' end with T4 polynucleotide kinase and [γ-^{32}P]ATP (1,000 Ci/mmol, Radiochemical Center, Amersham). Single end-labeled DNAs were prepared for sequence analysis by secondary cleavage with another restriction endonuclease.

Northern blot hybridization: 1×10^9 of HL60 cells either induced by TPA or uninduced were collected and poly(A)-containing RNA was prepared as described previously (13). Electrophoresis and hybridization were carried out as described by Thomas (14), with minor modifications (15).

RESULTS AND DISCUSSION

Several clones corresponding to the mRNAs specifically expressed during differentiation of HL60 cells by TPA were obtained (16). One clone designated as pHH81 harboring 180bp insert was used as a first probe to analyze the expression of corresponding mRNA during redifferentiation of HL60 cells. As indicated in Fig 1, mRNA with about 4.3kb in length was detected in the mRNAs obtained from induced HL60 cells, but not in uninduced cells. The mRNA seemed to be maximally expressed 30-60 min after addition of TPA and gradually diminished thereafter. So this mRNA is one of those newly synthesized during early differentiation of HL60 cells. In order to obtain more information about this mRNA, we tried to isolate clones which have longer inserts than pHH81 using its insert as a probe. One clone designated as pHH58 harboring 730bp of insert was obtained. Unexpectedly, Northern blot analysis revealed that additional 0.7kb mRNA was detected when ^{32}P-nick-translated cDNA insert of pHH58 was used as a hybridization probe. Moreover,

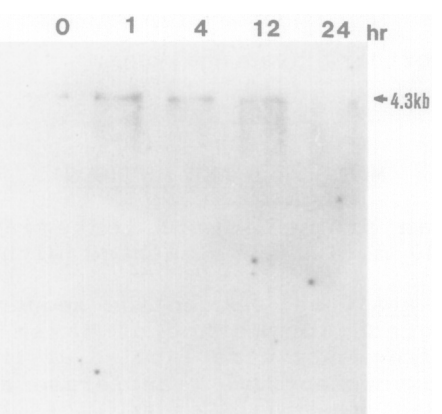

Fig. 1. Detection of 4.3kb mRNA with the insert of pHH81 as a probe. Poly(A) RNAs were isolated from HL60 cells at 0 (without TPA), 1, 4, 12 and 24 hr after addition of 10 ng/ml of TPA. 1 μg each of these RNAs was glyoxalated, electrophoresed on agarose gel and transfered to nitrocellulose filter. The filter was hybridized with ^{32}P-nick-translated cDNA insert prepared from PstI cleavage of pHH81. The arrow indicates the position of RNA with approximately 4.3kb which was determined using glyoxalated HindIII digests of λDNA.

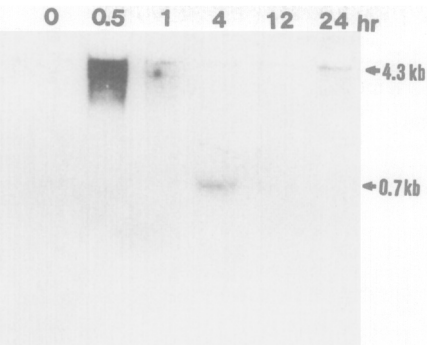

Fig. 2. Northern blot hybridization using the insert of pHH58 as a probe. Poly(A) RNAs were prepared and processed as described in Fig. 1 except RNA sample was also prepared from 0.5 hr-induced cells. The filter was hybridized with ^{32}P-nick-translated cDNA insert obtained by PstI cleavage of pHH58. The arrow indicates the position of RNA with approximately 4.3kb and 0.7kb which were determined using glyoxalated HindIII digests of λDNA or Sau3A digests of pBR322.

maximum expression of the 0.7kb RNA was observed 4 hr after addition of TPA, and the level of 0.7kb mRNA decreased dramatically thereafter, although 4.3kb mRNA decreased 3-5 fold and remained at this level for the following 20 hr (Fig. 2). This result indicated that the initiation of transcription of the two related mRNAs are differentially regulated. To elucidate the structural relationship between these two mRNAs, the nucleotide sequence of cDNA inserts of both pHH81 and pHH58 was determined. As indicated in Fig. 3, nucleotide sequence of the insert of pHH81 was quite identical to the 3' region of pHH58, and both clones contained the poly(dA-dT) tract derived from poly(A) sequence of mRNA. Restriction endonuclease cleavage map suggested and nucleotide sequence determination definitively demonstrated that the sequences of the clones pHH81 and pHH58 were derived from the same mRNA; the mRNA is the 4.3kb species, because only 4.3kb mRNA was detected using the insert of pHH81 as a probe. As depicted in the figure the sequence of the insert of pHH81 corresponded to the region 3' downstream of the PstI

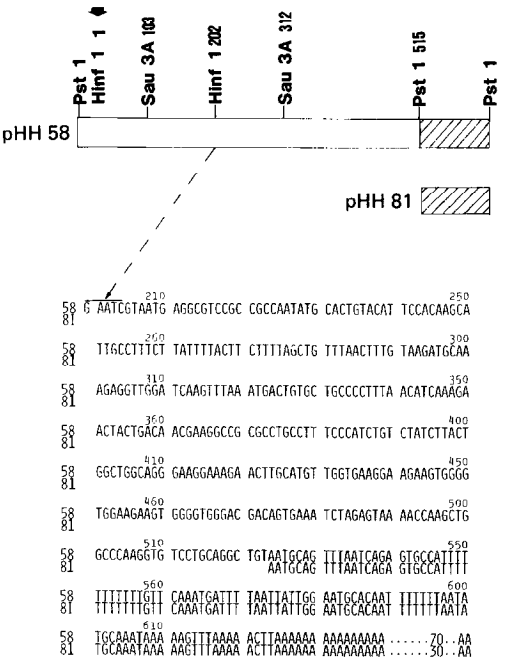

Fig. 3. Restriction endonuclease cleavage map and partial nucleotide sequence of pHH58 and pHH81. The restriction endonuclease cleavage map displays only relevant restriction endonuclease sites. Nucleotide residues are numbered in the 5' to 3' direction beginning with the nucleotide A of 5'-terminal nucleotide generated by HinfI cleavage as indicated by vertical arrow. The poly(dG)poly(dC) tails are not included in the map. DNA sequencing was carried out by the procedure of Maxam and Gilbert (12). DNA sequence of pHH81 begins with nucleotide residue number 525 of pHH58 insert.

site present in the clone pHH58. This was confirmed by Northern blotting using as probes 5' upstream region (probe a) or 3' downstream region (probe b) which were generated by PstI cleavage of the insert of pHH58. The result shown in Fig. 4 clealy demonstrated that 0.7kb mRNA was only detected when 5' upstream region from PstI cleavage site was used as a probe, on the other hand 4.3kb mRNA was detected with both DNA probes. Since these two closely related mRNAs were induced during early differentiation of HL60 cells by TPA, they might be involved in some early events in differentiation, Thus, further structural relationship between these two mRNAs is of great interest to elucidate: one possible attractive possibility is that 0.7kb

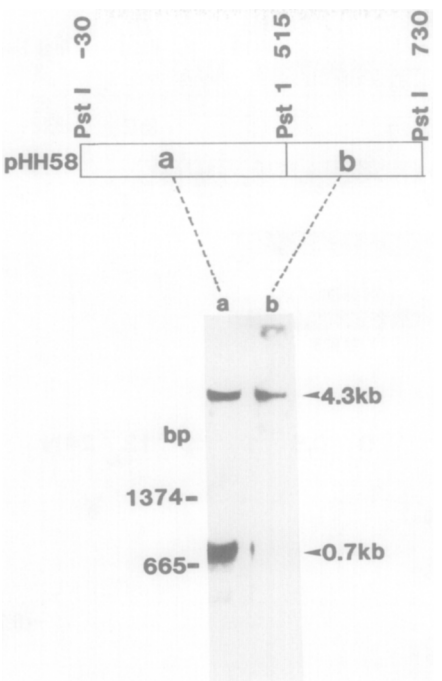

Fig. 4. Northern blot analysis of RNAs obtained from 4 hr-induced cells with probes of 5' upstream or 3' downstream regions generated by PstI cleavage of the insert of pHH58. 1 μg of poly(A) RNA from HL 60 cells harbested at 4 hr after addition of TPA was glyoxalated and electrophoresed on agarose gel followed by transfer to nitrocellulose filter. The filter was hybridized separately with ^{32}P-nick-translated 5'-upstream probe (a) or 3'- downstream probe (b) generated by PstI cleavage of pHH58. The arrow indicates the positions of RNA with approximately 4.3kb or 0.7kb determined as described in Fig. 1 and 2, and 1374 or 665 indicate the position of DNA fragments of glyoxalated Sau3A digests of pBR322.

mRNA could be an anti-sence transcript from the specific portion of the DNA from which 4.3kb sense mRNA is transcribed. In order to examine this possibility, Northern blotting analysis was carried out with a probe specifically designed to detect anti-sense transcript. The probe was prepared as described in Fig. 5A. The figure shows restriction endonuclease cleavage map of HinfI fragment of pHH58 and the direction of transcription of 4.3kb mRNA. The HinfI fragment with 778bp corresponds to the DNA spanning from the number 202 nucleotide in the pHH58 to the number 3362 nucleotide in pBR322 (16). After phosphatase treatment, 5' termini were labeled with [γ -^{32}P] ATP and

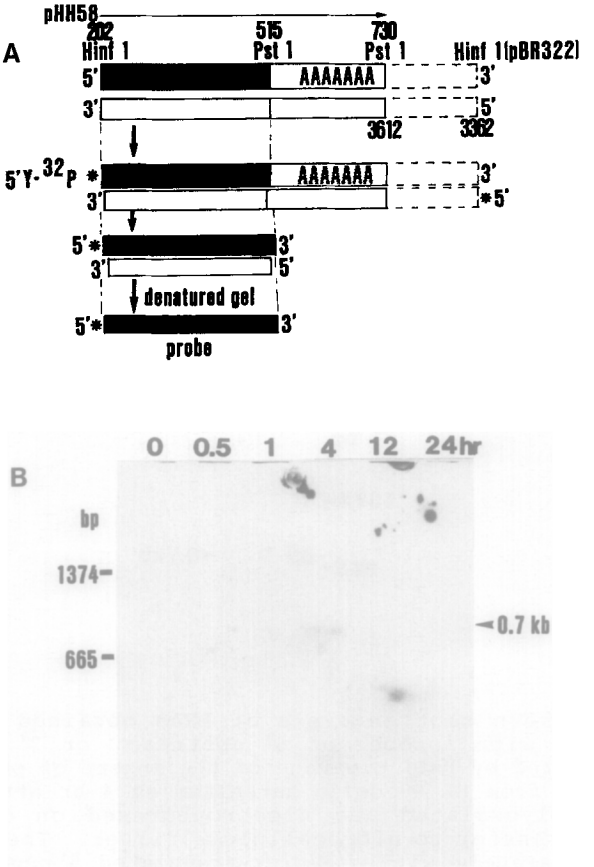

Fig. 5. Northern blot analysis detecting anti-sense RNA.
A. pHH58 was cleaved by restriction endonuclease HinfI, and 5' terminal phosphate of the fragment was removed by alkaline phosphatase. The fragment of 778bp was purified by polyacrylamide gel electrophoresis. Both termini were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by PstI cleavage which generates 313bp fragment labeled at 5' end of anti-sense strand.
B. Poly(A) RNAs were prepared and processed as described in Fig. 2. The filter was hybridized with the probe described in A. The arrow indicates the position of RNA with approximately 0.7kb as determined using 1374 and 665bp fragments of glyoxalated Sau3A digests of pBR322.

polynucleotide kinase, and the fragment was cleaved by PstI. The 313bp fragment generated from 5' half of the HinfI fragment was recovered and used as an anti-sense transcript-specific probe in Northern blot analysis. Fig. 5B shows that 0.7kb mRNA was hybridized with the probe, indicating that the 0.7kb RNA was in fact an anti-sense transcript from the same DNA segment as 4.3kb

mRNA is derived. The kinetics of its expression after addition of TPA was identical with those probed with nick-translated cDNA insert of pHH58. These data show that an anti-sense RNA is transiently synthesized at early stages of differentiation.

Biological significance of anti-sense poly(A) RNA in HL60 cells during redifferentiation by TPA is, however, unknown at present. Inouye and his colleagues (19, 20) showed that the expression of ompF RNA in E. coli was inhibited by micRNA which was a countertranscript of 5' upstream region of the ompC and complementary to the 5' end region of the ompF RNA. This was attributed to the translational inhibition of ompF RNA by making hybrid with a complementary RNA transcript. Furthermore, Coleman et al. (23) extended their study and showed that artificially induced anti-sense RNA (micRNA) system provides an efficient means to regulate the expression of specific prokaryotic genes. In bacteria, expression of several other species of anti-sense RNAs have been reported (20-22) which regulate the initiation of plasmid DNA replication by interfering with the function of primer RNAs. Izant and Weintraub (24, 25) raised the posibility that endogenous anti-sense regulation may be one of the normal components of gene regulation in eukaryotes. They have shown that artificially constructed anti-sense transcripts of the herpes simplex virus thymidine kinase (TK) gene can diminish the appearance of viral TK gene activity in mouse TK^- L cells. This experiment in turn suggests the possibility that our 0.7kb poly(A) RNA will be involved in the regulation of redifferentiation of HL60 cells into macrophages by hybridizing with 4.3kb sense mRNA. Melton (26) demonstrated that covering of 5' upstream region but not 3' downstream region of a mRNA by anti-sense RNA is necessary for the blockade of translation. Since 0.7kb RNA is in all probability complementary to 3' nontranslated region of 4.3kb

mRNA, it is still unknown whether translational inhibition such as by micRNA or artificial anti-sense RNA is involved in the function of 0.7kb mRNA. Early in 1977 Yanagi et al. (27) using SV40 DNA and recently Farnham et al. (28) using mouse dihydrofolate reductase (DHFD) gene have shown that anti-sense countertranscripts are synthesized, but they are confined in the nucleus. Since the 0.7kb RNA was detected in cytoplasmic poly(A) RNA fraction, it should be functional in somewhat different way from that of the SV40 or DHFR nuclear countertranscripts. Further analyses are currently under way to elucidate functional relationship of these two mRNAs in redifferentiation of HL60 cells by TPA.

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