A Single 3.7-Kilobase Messenger RNA Hybridizes to Immediate-Early Promoter-Enhancer of Human Cytomegalovirus in HL-60 and Acute Myeloid Leukemia Cells

Zee-Fen Chang, Duen-Yi Huang, Chiow-Jiau Lin

Department of Biochemistry, Chang Gung Medical College, Tao-Yuan, Taiwan, Republic of China

Abstract

Expression of a mRNA cross-hybridized to human cytomegalovirus immediate-early gene promoter-enhancer was detected in the human promyelocytic leukemia cell line HL-60. The 0.6 kilobase of NruI/SacI DNA fragment of eukaryotic expression vector pCDM8 representing human cytomegalovirus immediate-early gene promoter-enhancer was used as the probe to hybridize with polyadenylated RNA by the Northern blot analysis. A 3.7-kilobase strand of polyadenylated RNA was visualized in the cytoplasmic fraction of HL-60 promyelocytes. In contrast, other human hematopoietic cell lines, hepatoma cells, and normal human fibroblasts did not show such a transcript by cross-hybridization. This transcript was called CMVE RNA. The expression of CMVE mRNA was also detectable in the fresh blast cells from patients with acute myeloid leukemia, and particularly from a patient with acute myeloid leukemia of the M3 type. Taken together, these findings suggest that the CMVE RNA-encoded gene plays an important role in the pathogenesis of acute promyelocytic leukemia.

Introduction

HCMV, a member of the human herpesvirus group, is capable of producing a permissive or latent infection in the human host. It is well established that HCMV IE promoter-enhancer has a very high basal activity in a wide range of uninfected host cells (1). This characteristic of the HCMV IE promoter-enhancer has led to its use in mammalian cell expression vector such as pCDM8 (2) and $\gamma$H3M (3). In this study, using a 32P-labeled CDM8 plasmid to hybridize with poly(A)+ RNA isolated from HL-60 cells, we have found that the proliferating HL-60 cells contain mRNA that could cross-hybridize with pCDM8 vector by Northern blot analysis. The 0.6-kilobase fragment (NruI/SacI) of the pCDM8, covering 98% of the sequence of HCMV immediate-early promoter-enhancer (2), has been found to be fully responsible for the hybridizable signal. This transcript was called CMVE RNA. Furthermore, we have demonstrated that the expression of the 3.7-kilobase CMVE RNA is very specific in the proliferating HL-60 cells and down-regulated in the differentiating HL-60 induced by TPA (4). The presence of CMVE RNA can also be detected in the fresh leukemic blast cells from patients with AMLs.

Materials and Methods

Cells and Cultures. All cell lines were obtained from the American Type Culture Collection and stored as frozen stocks in liquid nitrogen. Cells were maintained in the medium specified by the supplier at 37°C in 5% CO2. HL-60 cells were diluted to $2 \times 10^6$ cells/ml in fresh medium for the induction experiment. TPA (Sigma) was used at a final concentration of 30 nm. Fresh leukemic cells were obtained from AML patients at Chang Gung Memorial Hospital. AMLs were classified according to French-American-British classification (5). The leukemic blast cells were prepared from the peripheral blood of AML patients by centrifugation on a Ficoll-Paque gradient (density, 1.097).

Total RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from cells using a acid-ified guanidinium isothiocyanate-polyethylene glycol-sodium chloride extraction procedure described by Chomczynski and Sacchi (6). Polyadenylated RNA was prepared from total or cytoplasmic RNA by affinity chromatography on an oligodeoxythymidylate cellulose column (8). RNAs separated by formaldehyde gel electrophoresis were subsequently transferred to nitrocellulose paper (Schleicher & Schuell), and hybridized to appropriate DNA probes as described by Thomas (9). The hybridization buffer consisted of 50% (v/v) formamide, 5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2.5X Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.8). 200 µg of denatured salmon sperm DNA per ml, and 10⁶ cpm of the probe per ml. After 24 h of hybridization at 42°C, the nitrocellulose membrane was washed by 2X SSC plus 0.1% SDS for 15 min at room temperature twice and 0.2X SSC plus 0.1% SDS at 58°C for 30 min twice. The 0.6 kilobase of SacI/NruI fragment of pCDM8 was gel-purified and labeled with [α-32P]dCTP by random primer to $1 \times 10^8$cpm/µg as a probe (10). The 2.0-kilobases of PstI fragment of the chicken β-actin cloned in pGEM 2 (11) were used as an internal control.

Preparation of Radioactively Labeled Single-Stranded RNA Probes. The NruI/SacI fragment was cloned into pGEM5Z (Promega). The plasmid was then linearized with the suitable restriction enzyme for the production of riboprobe. The labeled RNA (specific activity, 8 x 10⁴cpm/µg of template DNA) was prepared by the in vitro transcription reaction (12, 13). The hybridization was carried out at 52°C and washed as described previously, except that the nitrocellulose membrane was further washed in 0.05% sodium pyrophosphate at 45°C overnight.

Genomic DNA Analysis. Nuclei were prepared from HL-60 and HepG29P cells and high molecular weight genomic DNA was isolated from nuclei pellets (14). The DNA samples were digested with restriction endonucleases according to conditions recommended by the manufacturer (Promega). Fragments were then separated on 1% agarose gels, transferred to nitrocellulose paper, and hybridized with the radio-labeled SacI/NruI DNA fragment of pCDM8 as described above.

Results

Cross-Hybridization of HCMV IE Promoter-Enhancer DNA Fragment with Poly(A+) RNA in the Proliferating but not in TPA-treated HL-60 Cells. The Northern blot of poly(A)+ RNA isolated from the proliferating HL-60 human promyelocytic leukemia cells was hybridized with the 32P-labeled fragment
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(Nrul/Sacl) of the pCDM8 plasmid. A 3.7-kilobase RNA transcript could be clearly detected in the proliferating HL-60 cells on the autoradiogram (Fig. 1). Since the washing condition for the Northern blot was relatively stringent (2x SSC for 30 min twice; 0.2x SSC at 58°C for 30 min twice), it is conceivable that specific homologous sequences are present between this mRNA transcript and the DNA fragment (Nrul/Sacl) of the pCDM8 plasmid. This 3.7-kilobase RNA transcript was named CMVE mRNA, implying that it contains a sequence homologous with CMV enhancer. Moreover, the hybridization signal disappeared when the cells were treated with TPA for 16 h (Fig. 1), suggesting that the expression of CMVE RNA was down-regulated by TPA in HL-60 cells.

Determination of Polarity of Sequence. The HCMV IE promoter-enhancer fragment was cloned into the vector pGEM 5Z (Promega), which contains SP6 and T7 promoter on each side of the insert. The inserted sequence can be transcribed in vitro from both directions (Fig. 2A). The two RNA probes thus derived (SP6 and T7 probes) were hybridized to the Northern blots of poly(A)+ RNA from the proliferating and TPA-treated cells separately. When SP6 RNA probe was used, a 3.7-kilobase transcript of the total poly(A)+ RNA from the proliferating HL-60 cells was detected (Fig. 2B). No hybridization with T7 riboprobe was observed. This result indicated that the polarity of the 3.7-kilobase RNA sense transcript was the same as that of the HCMV IE promoter-enhancer sequence. The slight hybridization signal at 28S and 18S might arise from the presence of a GC-rich region of HCMV enhancer riboprobe.

CMVE RNA Is not a Heterogeneous Nuclear RNA. To ascertain whether the 3.7-kilobase CMVE RNA is a mature mRNA, the total cellular poly(A)+ RNA and the cytoplasmic poly(A)+

RNA isolated from the proliferating HL-60 cells were compared and examined for the appearance of the CMVE RNA (Fig. 3). All of the CMVE RNA transcripts appeared in the cytoplasmic fraction, indicating that the CMVE RNA transcript was a mature mRNA.

Expression of CMVE mRNA in Human Cell Lines and AML Cells. A number of cell lines of myeloid, lymphoid, and erythroid origin as well as normal human diploid fibroblast and hepatoma cell line were examined for the expression of this CMVE RNA transcript. Poly(A)+ RNAs were prepared from the following cell lines: KG-1, a myeloid precursor line; K562, a cell line derived from a patient with chronic myelogenous leukemia but which has some erythroid precursor characteristics; U937, a human monocytoid cell line; Molt 4, an immature T-cell line of lymphoid origin; Raji, a Burkitt lymphoma cell line; HepG2P9, a hepatoma cell line derived from HepG2; IMR-90, a human embryonic lung fibroblast; and HDF, a human foreskin fibroblasts. Unlike results in HL-60 cells, CMVE RNA transcript could not be detected in other cell lines (Fig. 4A). Since HL-60 is a promyelocytic cell line derived from leukocytes of an AML patient, we then examined the expression of CMVE mRNA in other AMLs and one CMMOL that was recently transformed into an acute leukemia. The CMVE RNA was detected in all six of the AMLs examined (Fig. 4B). The hybridization signal was relatively weak in the CMMOL cells upon transformation to AML. M3 type AML expressed the
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highest level of CMVE RNA as compared to HL-60, M1, M2, and M4 types.

Genomic DNA Analysis. We compared the pattern of the digested genomic DNAs from the proliferating HL-60 cells and HepG2P9 cells that hybridized with HCMV IE promoter-enhancer by Southern blot analysis. It appeared that none of the digested genomic DNA bands detected by NruI/SacI fragment of pCDM8 from HL-60 and HepG2P9 cells was similar in size (Fig. 5). This result indicated that the location of a genomic sequence that shared homology with HCMV IE promoter-enhancer depended on the cell lines, which in turn might determine the expression of CMVE mRNA. Furthermore, the hybridization signal of the DNA fragments isolated from HepG2P9 cells was quite faint compared to that detected in

Fig. 3. Northern blot analysis of total and cytoplasmic poly(A)+ RNA of HL-60 cells. Cytoplasmic poly(A)+ RNA fraction was isolated from the proliferating HL-60 cells. Five µg each of total cellular and cytoplasmic poly(A)+ RNA was analyzed by Northern blot hybridized with SP6 riboprobe. The same blot was rehybridized with 32P-labeled β-actin probe. kb, kilobases.

Fig. 4. (A) Detection of CMVE mRNA in different human cell lines. Lanes contained poly(A)+ RNA from: Lane 1, HL-60; Lane 2, Raji; Lane 3, Molt-4; Lane 4, KG1; Lane 5, U937; Lane 6, K562; Lane 7, HepG2P9; Lane 8, IMR-90 normal human embryonic lung fibroblasts; Lane 9, HDF normal human foreskin diploid fibroblasts. Five µg of total cellular poly(A)+ RNAs were prepared from the proliferating cells. The Northern blot was hybridized with the SP6 riboprobe. The same blot was rehybridized with 32P-labeled β-actin probe. (B) CMVE mRNA expression in human AML breast cells. Northern blot analysis was performed as described in "Materials and Methods." Each lane contained 5 µg of total poly(A)+ RNA isolated from AML patients of M4 type (Lane 1), M2 type (Lane 2), M3 type (Lane 3), M4 type (Lane 5), M2 type (Lane 6), and M1 type (Lane 7). Lane 4 contained RNA isolated from CMMOL patient transformed to AML M4 type. One lane contained the same amount of RNA isolated from HL-60 cells for comparison. The blot was hybridized with SP6 riboprobe and rehybridized with 32P-labeled β-actin probe. The autoradiographic exposure times for 32P-labeled β-actin probe and 32P-labeled riboprobe were both 2 h. kb, kilobases.
HL-60 cell. This could be due to the divergence of sequence that could cross-hybridize to HCMV IE promoter-enhancer sequence in HepG2P9 cells. Alternatively, it is possible that the copy number of the cross-hybridized sequence of HCMV IE sequence in HepG2P9 cells. Alternatively, it is possible that the copy number of the cross-hybridized sequence of HCMV IE promoter-enhancer region and the genomic DNA sequences of the HL-60 cells, but much less hybridization in other human cell lines (Fig. 5). This indicates that the specific DNA sequences present in the HL-60 genome share significant homology with HCMV immediate-early promoter-enhancer. In turn, this may explain why CMVE mRNA could be detected in HL-60 cells but not in other cell lines used in this study. The mechanism that endowed HL-60 cells with the DNA sequences homologous to HCMV IE promoter-enhancer remains to be investigated.

The expression of CMVE mRNA transcript has also been presented in the peripheral blood samples of AML patients. Six of the 7 AML samples, (ranging from M1 to M4) significantly expressed this transcript (Fig. 4B). The hybridization signal of the RNA sample from the CMMOL patient transformed to the M4-type AML was evident only after longer exposure of the Northern blot for the autoradiogram. In one case of M3 type AML, CMVE mRNA was highly expressed. M3-type AML generally represents acute promyelocytic leukemia. The expression of CMVE mRNA only in HL-60 cells and abundantly in M3-type AML indicates that the expression of CMVE mRNA may be very specific to the neoplastic promyelocytes. Acute promyelocytic leukemia has been characterized by a predominance of malignant promyelocytes that carry a reciprocal translocation between the long arms of chromosomes 15 and 17, t(15;17)(q22;11.2-q12) (20). Whether the expression of CMVE mRNA is the result of the genomic rearrangement remained further to be seen. Since the expression of the CMVE mRNA is down-regulated by TPA, it is very likely that its encoded gene is highly regulated. We are currently cloning the cDNA of CMVE mRNA, which should allow us to carry out more studies to understand its physiological role.

Discussion

This report has demonstrated the cross-hybridization between the sequence of immediate-early promoter-enhancer of HCMV and a 3.7-kilobase poly(A)+ RNA present in the cytoplasmic fraction of HL-60 cells and AML blast cells. Such a finding raises the questions of whether CMVE RNA is an already known mRNA which is preferentially expressed in human promyelocytic leukemia cells and whether the cross-hybridization is due to the GC richness of the probe. By computer-assisted alignment (15), we did not find any significant homology between HCMV IE gene promoter-enhancer and other known human complementary DNA sequences data stored in the GenBank, suggesting that the sequence of the CMVE mRNA-encoded gene in human cells may have never been identified. It has been reported that the genome of HCMV can cross-hybridize with v-myc oncogene and genomic DNA of human placental tissues. Such cross-hybridizations were then found to be the fortuitous consequence of the high GC-rich content of these DNAs regions (16, 17). The HCMV IE enhancer-promoter also contains some GC-rich regions; however, by using bidirectional riboprobes (Fig. 2B) we found that only one direction of riboprobe could hybridize with CMVE RNA. This indicates that the cross-hybridization is not merely due to GC content of the probe and the transcript. The findings that HL-60 cells have low susceptibility to HCMV infection (18) and that the major RNA transcripts produced by HCMV infection are not similar to CMVE mRNA nor do these HCMV immediate-early RNA transcripts contain enhancer sequences (19) exclude the possibility that the detection of this 3.7-kilobase RNA transcript is due to HCMV infection. All together, we have concluded that the hybridization is due to the presence of specific homologous sequences.

By genomic DNA analysis, we observed intense cross-hybridization between the HCMV IE promoter-enhancer region and the genomic DNA sequences of the HL-60 cells, but much less hybridization in other human cell lines (Fig. 5). This indicates that the specific DNA sequences present in the HL-60 genome share significant homology with HCMV immediate-early promoter-enhancer. In turn, this may explain why CMVE mRNA could be detected in HL-60 cells but not in other cell lines used in this study. The mechanism that endowed HL-60 cells with the DNA sequences homologous to HCMV IE promoter-enhancer remains to be investigated.

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Acknowledgments

We are grateful to Dr. L-Y. Shih at Chang Gung Memorial Hospital, Taiwan, for collecting samples from AML patients. We thank C-Y. Zheng for her excellent technical help and Dr. Brian Seed for providing pCDM8 vector. We express our sincere gratitude to Drs. Ming-Ta Huang and Daniel T. Y. Chiu for their valuable suggestions for manuscript preparation.

References

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