Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a

Amit Sharma, Manish Kumar, Jyotirmoi Aich, Manoj Hariharan, Samir K. Brahmachari, Anurag Agrawal, and Balaram Ghosh

Molecular Immunogenetics Laboratory and G. N. Ramachandran Knowledge Centre for Genome Informatics, Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India

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IL-10 is a key regulator of the immune system that critically determines health and disease. Its expression is finely tuned both at the transcriptional and posttranscriptional levels. Although the importance of posttranscriptional regulation of IL-10 has been previously shown, understanding the underlying mechanisms is still in its infancy. In this study, using a combination of bioinformatics and molecular approaches, we report that microRNA (hsa-miR-106a) regulates IL-10 expression. The hsa-miR-106a binding site in the 3′ UTR of IL10 has been identified by site-directed mutagenesis studies. Also, the involvement of transcription factors, Sp1 and Egr1, in the regulation of hsa-miR-106a expression and concomitant decrease in the IL-10 expression, has also been demonstrated. In summary, our results showed that IL-10 expression may be regulated by miR-106a, which is in turn transcriptionally regulated by Egr1 and Sp1.

IL-10 is a key orchestrator of the immune system that has been shown to be antiinflammatory in many model systems (1). It is secreted by various cell types like different T cell subsets, macrophages, dendritic cells (DC), B-cells, mast cells, and eosinophils. It mediates a plethora of immunoregulatory events such as maturation and activation of macrophages and DC, expression of MHC-II and B-7; activation of T cells, synthesis of cytokines, and antibody production (2, 3). Also, dysregulation of IL-10 expression leads to various immunological diseases, such as cancer, rheumatoid arthritis, asthma, infectious disorders, etc. (2). Therefore, it is likely that IL-10 expression is tightly regulated.

Regulation of IL-10 expression has been studied at the transcriptional and posttranscriptional levels. Although Sp1 and Sp3 have been found to regulate transcription, it has been shown that IL10 mRNA is constitutively transcribed in many cells; however, the availability of its protein level is significantly determined by posttranscriptional mechanisms (4, 5). AU-rich elements (ARE) in the 3′ UTR of mouse IL10 that lead to the degradation of its mRNA have been shown (6). Half life of IL10 mRNA in normal melanocytes is much shorter than in melanoma cell lines (7). Also, genetic variations in the 3′ UTR of IL10 have been shown to be associated with IL-10 levels that could lead to disease pathogenesis (8, 9). Therefore, a pellucid understanding of the posttranscriptional regulation of IL10 will be of scientific and clinical significance.

A growing class of noncoding RNAs called microRNAs (miRNAs) is involved in posttranscriptional regulation of genes (10). MicroRNAs have been reported to modulate hematopoietic lineage differentiation (11), angiogenesis (12), cell adhesion (13), etc., indicating that they could have important roles in numerous biological processes (14). There is also a growing body of literature supporting the potential role of miRNAs in regulation of inflammatory processes (15). The importance of IL-10 in the orchestration of the immune response, and the strong evidence for its posttranscriptional regulation, makes it an attractive candidate for miRNA mediated regulation of inflammation.

Here, we report the identification of a miRNA, hsa-miR-106a, that regulates IL-10 expression. We also find that Sp1 and Egr1 have an important role in hsa-miR-106a transcription and, thus, indirectly regulate the expression of IL-10 posttranscriptionally.

Results

Prediction of miRNA Regulators of IL-10. To predict the miRNA that may regulate IL-10, we used a consensus approach by employing 3 different miRNA target prediction software, as described in Materials and Methods. We found that 8 miRNAs have potential binding site in the 3′ UTR of IL10 (Table 1). Also, using miRex, we found that 5 miRNAs (namely hsa-miR-106a, miR-106b, miR-20a, miR-20b, and miR-93) were expressed in cells of lymphoid and myeloid origin, which are known to express IL-10.

Identification of hsa-miR-106a As a Regulator of IL-10 Expression. To identify the miRNA(s) that may be involved in the regulation of IL-10, initially Raji cell line was chosen, because it constitutively expresses high level of IL-10 and, thus, presumably may have lower level of regulatory miRNA(s). When these cells were transfected with the 5 predicted miRNA oligonucleotides, hsa-miR-106a oligonucleotide significantly down-regulated the expression of IL-10 present in the culture supernatant (Fig. 1A). Interestingly, hsa-miR-106b, which differs from hsa-miR-106a in only 2 nucleotide positions, also affected IL-10 expression, albeit at a lower level. In contrast, transfection with other 3 miRNA or cel-miR-67, a miRNA from Caenorhabditis elegans having no sequence identity with the predicted human miRNAs (used as negative control), had no effect on the IL-10 expression (Fig. 1A). Also, when increasing concentration of hsa-miR-106a was used, IL-10 expression was found to be reduced in a dose-dependent manner (Fig. 1B).

To check whether hsa-miR-106a affects the stability of IL10 mRNA, RT-PCR was performed by using RNA prepared from Raji cells transfected with increasing concentration of hsa-miR-106a oligonucleotide. It was observed that it down-regulated IL10 mRNA in a concentration-dependent manner (Fig. 1C), whereas cel-miR-67 had no effect. Thus, it is likely that hsa-miR-106a decreases IL-10 expression by degrading its mRNA.


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To confirm the involvement of miR-106a further, we chose Jurkat cells that produce low basal level of IL-10, and, thus, may produce higher level of hsa-miR-106a. When Jurkat cells were transfected with increasing concentration of anti-hsa-miR-106a oligonucleotide and IL-10 levels were measured in the culture supernatant, a dose-dependent increase in the levels of IL-10 was observed (Fig. 1D). In contrast, a random oligonucleotide had no effect on IL-10 expression. Thus, these experiments demonstrate that hsa-miR-106a regulates IL-10 expression.

Expression of hsa-miR-106a and Its Involvement in IL-10 Regulation in Different Cells. To specifically detect the expression of hsa-miR-106a, we standardized Northern blotting conditions so that we specifically detect hsa-miR-106a, not hsa-miR-106b (Fig. 2A). Next, when blot was performed using RNA prepared from A549, Jurkat, Raji, HeLa, Hep-G2, and THP-1 cells, hsa-miR-106a was found to be expressed significantly in Jurkat, Raji, and THP-1 cells (Fig. 2B). However, Hep-G2, HeLa, and A549 cells expressed lower amounts of hsa-miR-106a. To correlate the expression of hsa-miR-106a with 3' UTR regulatory activity, Jurkat, Raji, THP-1, and A549 cells were transfected with pMIR-REPORT-IL10 3' UTR (intact). A substantial fall in luciferase activity was observed in Jurkat, Raji, and THP-1 cells. In comparison, the decrease in A549 cells was not profound. Thus, the down-regulatory effect of IL10 3' UTR was found to be correlated with the levels of mature hsa-miR-106a present in these cells (Fig. 2C).

Confirmation of Target Site for hsa-miR-106a in IL10 3' UTR. Our bioinformatics analysis predicted the binding of hsa-miR-106a to the 3' UTR of IL10 in a region encompassing +4451 to +4478 bases (Fig. 3A). When luciferase reporter vector containing the intact 3' UTR was transfected in Jurkat or Raji cells, a significant down-regulation in the luciferase expression in both cells with respect to control was observed (Fig. 3B). This down-regulation was not seen in mutant 3' UTR construct lacking the predicted 27 base hsa-miR-106a binding site. Also, when A549 cells, which expresses minimal level of hsa-miR-106a, were cotransfected with hsa-miR-106a oligonucleotide and pMIR-REPORT-IL10 3' UTR (intact), we observed >2-fold reduction in luciferase activity.

Table 1. A list of predicted miRNAs (in the decreasing order of binding free energy) having potential binding sites in the 3’ UTR of IL10

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Start site</th>
<th>End site</th>
<th>Binding free energy, Kcal/mol</th>
<th>Expression status in lymphoid and myeloid origin cells</th>
</tr>
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<tbody>
<tr>
<td>hsa-miR-20b.MIMAT0001413</td>
<td>625</td>
<td>647</td>
<td>−24.32</td>
<td>Yes</td>
</tr>
<tr>
<td>hsa-miR-640. MIMAT0003310</td>
<td>654</td>
<td>670</td>
<td>−24.16</td>
<td>No data</td>
</tr>
<tr>
<td>hsa-miR-93. MIMAT0000093</td>
<td>625</td>
<td>646</td>
<td>−23.88</td>
<td>Yes</td>
</tr>
<tr>
<td>hsa-miR-20a. MIMAT000075</td>
<td>625</td>
<td>647</td>
<td>−22.21</td>
<td>Yes</td>
</tr>
<tr>
<td>hsa-miR-106b. MIMAT0003310</td>
<td>627</td>
<td>647</td>
<td>−20.77</td>
<td>Yes</td>
</tr>
<tr>
<td>hsa-miR-597. MIMAT0003265</td>
<td>249</td>
<td>270</td>
<td>−20.52</td>
<td>No data</td>
</tr>
<tr>
<td>hsa-miR-372. MIMAT0000724</td>
<td>619</td>
<td>646</td>
<td>−20.46</td>
<td>No data</td>
</tr>
<tr>
<td>hsa-miR-106a.MIMAT0003310</td>
<td>621</td>
<td>647</td>
<td>−20.35</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Fig. 1. Identification of hsa-miR-106a as a regulator of IL-10 expression. (A) Raji cells were transfected with or without the 5 candidate miRNA oligonucleotides individually, or with a negative control oligonucleotide, cel-miR-67 (having no sequence identity with any known human miRNAs); 24 h after transfection, IL-10 was measured from the culture supernatants by ELISA, and plotted as fold change in IL-10 with respect to untransfected control. (B) Raji cells were transfected without or with increasing concentrations of hsa-miR-106a or cel-miR-67, and IL-10 level was measured, as described in Materials and Methods. (C) Raji cells were transfected with or without increasing concentrations of hsa-miR-106a or cel-miR-67 as described in B, and RNA was prepared followed by RT-PCR by using IL10 specific primers. GAPDH RT-PCR was performed as loading control. Representative of 3 independent experiments. (D) Jurkat cells were transfected with or without increasing concentration of anti-hsa-miR-106a oligonucleotide or a negative control oligonucleotide followed by IL-10 measurement in the culture supernatant by ELISA, 24 h after transfection. Results in A, B, and D are presented as mean ± SE.
expression (Fig. 3C). However, cotransfection with cel-miR-67 did not have any appreciable effect on luciferase expression. Most importantly, when hsa-miR-106a was cotransfected along with pMIR-REPORT-IL10 3’ UTR (mutant), there was minimal reduction in the luciferase expression. Also, cotransfection of pMIR-REPORT-IL10 3’ UTR (mutant) with cel-miR-67 had no effect on luciferase expression (Fig. 3C). These experiments indicate that mature hsa-miR-106a regulates IL-10 expression by interacting with its 3’ UTR.

Transcriptional Regulation of hsa-miR-106a. Because the level of hsa-miR-106a was found to vary in different cell lines (Fig. 2B), and this variation could be due to differential regulation of its expression, we sought to investigate the transcriptional regulation of hsa-miR-106a locus present on the X chromosome. Thus, a region encompassing 1 kilobase upstream of the putative transcription start site (TSS) of hsa-miR-106a was analyzed by using Transfac Alibaba prediction software (16). Two transcription factors, Egr1 and Sp1, were predicted to bind to this region (Fig. 4A). When this 1 kilobase putative promoter region was cloned upstream of the luciferase gene (pGL3 basic, a promoter less vector) and transfected in Jurkat cells, we observed a 4-fold induction in the luciferase expression (Fig. 4B); thus, indicating that the putative promoter sequence possessed regulatory activity.

To establish the binding of putative transcription factors Sp1 and Egr1, we determined the levels of these proteins using Western blotting of nuclear extracts prepared from unstimulated or stimulated Jurkat cells (PHA/PMA combination for 2 h). Although the basal level of Egr1 in Jurkat cells was undetectable, there was a significant increase after stimulation with PHA/ PMA combination (panel 1 in Fig. 4C). However, the level of Sp1 was unaffected by stimulation (panel 2). To demonstrate the binding of Sp1 and Egr1, EMSAs were performed by using end-labeled oligonucleotide probe from hsa-miR-106a potential promoter region (~285 to ~317 bases) and nuclear extract from unstimulated (for Sp1) Jurkat cells. Out of the shifted bands (Fig. 4D, lane 2), our cold chase experiments with miR-106a promoter oligonucleotide (lane 3), irrelevant oligonucleotides NF-κB (lane 4), and Sp1 oligonucleotide (lane 5) indicated the uppermost band (with an arrow) could be Sp1. The specificity of Sp1 band was further confirmed by antibody experiments, where anti-Sp1 antibody abolished this uppermost band (lane 6), whereas anti-Sp3 (lane 7) or normal goat IgG (lane 8) could not.

For Egr1, when nuclear extract from stimulated Jurkat cells was incubated with labeled hsa-miR-106a promoter oligonucleotide, only 1 shifted band was observed (Fig. 4E, lane 3). However, the band was absent when unstimulated nuclear extract was used (Fig. 4E, lane 2). Specificity of the shifted band was confirmed by cold chase with excess of Egr consensus oligonucleotide (lane 4), which abolished the specific band. However, cold chase with Egr mutant oligonucleotide was not able to chase the specific band (lane 5). This observation was further confirmed by antibody super shift experiments, where antibody against Egr1 was able to shift the band (lane 6), whereas non-specific IgG did not (lane 7). These experiments indicate that both Sp1 and Egr1 bind at an overlapping sequence in the promoter region, and could regulate the transcription of hsa-miR-106a.

To determine the effect of induced Egr1 expression on hsa-miR-106a level, Jurkat cells were stimulated with PHA/ PMA combination for different time intervals, and Northern blotting was performed. It showed that there was an increase in the levels of hsa-miR-106a up to 8 h (Fig. 4F), and its levels

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Fig. 2. Expression of hsa-miR-106a and its involvement in IL-10 regulation in different cells. (A) DNA oligonucleotides of hsa-miR-106a, hsa-miR-106b, and a random sequence of equal length were run on an acrylamide gel and probed with hsa-miR-106a radiolabeled probe. The probe was able to specifically discriminate between 2 forms of the miRNA. (B) Northern blotting was performed with total RNA from A549, Jurkat, Raji, Hela, HepG-2, and THP-1 cells by using radiolabeled probe specific for hsa-miR-106a. The membrane was stripped, and Northern blotting was performed for U6, which was used for normalization. Lower panel indicates U6 band. Representative of 3 independent experiments. (C) The pMIR-REPORT-IL10 3’ UTR (intact) was transfected in A549, Jurkat, Raji, and THP-1 cells, and luciferase expression was measured. Relative fold change in luciferase activity for each cell line was plotted with respect to pMIR-REPORT. A dark bar indicates cells transfected with pMIR-REPORT vector, whereas a light bar indicates cells transfected with pMIR-REPORT-IL10 3’ UTR (mutant). Results are presented as mean ± SE.

Fig. 3. Confirmation of target site for hsa-miR-106a in IL10 3’ UTR. (A) Schematic representation of IL10 3’ UTR indicating the binding site of hsa-miR-106a as predicted. This region encompassing ~4451 to ~4478 bases was deleted by PCR based site-directed mutagenesis. (B) Luciferase reporter vectors pMIR-REPORT-IL10 3’ UTR (intact) or pMIR-REPORT-IL10 3’ UTR (mutant) were transfected in Jurkat or in Raji cells. The fold change in relative luciferase activity was plotted. Dark and light bars indicate luciferase activity in Jurkat cells and Raji cells, respectively. (C) A549 cell line was cotransfected with hsa-miR-106a oligonucleotide or cel-miR-67 along with either pMIR-REPORT-IL10 3’ UTR (intact) or pMIR-REPORT-IL10 3’ UTR (mutant). The luciferase activity relative to pMIR-REPORT-IL10 3’ UTR (intact) was plotted. Results in B and C are presented as mean ± SE for 3 independent experiments.
miR-106a preceded the decline of mature hsa-miR-106a (compare 4 versus 8 h). These results indicate that induced Egr1 increased the expression of hsa-miR-106a.

Activation of hsa-miR-106a Promoter Regulates IL-10 Expression. To correlate Egr1 mediated activation of hsa-miR-106a promoter with IL-10 expression, Jurkat cells were stimulated with PMA, PHA, or PMA/PHA combination for 2 h, and expression of Egr1 was detected by Western blotting (Fig. 5A). It was observed that although PMA (50 ng/mL) alone did not affect Egr1 levels, PHA (5 μg/mL) was able to induce it. However, it was the combination of both PMA/PHA that was able to maximally induce Egr1 levels (Fig. 5A). Next, to determine the level of induced hsa-miR-106a, Jurkat cells were treated with PMA, PHA, or PMA/PHA combination for 8 h. Stimulation with PMA/PHA combination but not PMA alone induced hsa-miR-106a expression (Fig. 5B). This upregulation could be inhibited by specifically knocking down Egr1 expression by using Egr1 siRNA before stimulation (Fig. 5 C and D).

To correlate the level of Egr1 induction with the levels of IL-10, Jurkat cells were treated with PHA, PMA, or PHA/PMA together for 24 h, and IL-10 was measured in the culture supernatant by ELISA (Fig. 5E). It was observed that Jurkat cells induced with PMA alone resulted in 3.5-fold increase in IL-10 expression (Fig. 5E, lane 3), whereas PHA alone or PMA/PHA combination significantly reduced IL-10 levels (Fig. 5E, lanes 2 and 4). Thus, the level of IL-10 was inversely correlated with the level of Egr1-induced hsa-miR-106a expression.

Discussion

The posttranscriptional regulation of IL-10 is a subject of paramount clinical interest because of the crucial role of IL-10 as a modulator of inflammation. In this study, we found that hsa-miR-106a has a key role in regulating IL-10 expression. We provide a combination of in silico and in vitro evidence including a series of transfection experiments with various potential miRNAs in support of this conclusion.

Our finding of differential level of hsa-miR-106a (Fig. 2B) in various cells supports miRNA mediated control of IL-10 is
operational in cells that actively produce it. Recently, it has been demonstrated that regulatory T (Treg) cells have a miRNA profile distinct from conventional CD4 T cells, and some miRNAs, including hsa-miR-106a, were found to be down-regulated (17). In our study, the identification of hsa-miR-106a that regulates IL-10 expression further supports this observation. It can also be seen that there is a thin line between regulation and dysregulation of IL-10 expression. It is observed that in 46% of human T cell leukemias, pri-miR-106–363 cluster is overexpressed (18), presumably modulating IL-10 expression that could promote clonal expansion and leukemic cell survival. Our current results with the promoter revealed the presence of overlapping binding sites for Sp1 and Egr1 that may have potential regulatory function for hsa-miR-106a, a member of this cluster. Sp1 is a well characterized ubiquitous transcription factor that regulates a vast array of genes, including IL-10 (4, 5). The early growth response gene product (Egr1) is a zinc finger transcription factor with a characteristic brisk kinetics of induction. It can either positively or negatively regulate gene transcription in response to stimulation, because Egr1 contains both transactivation and repression domains (19). It has been proposed that Egr1 might be up-regulated in response to environmental challenge. Overlapping binding sites for Egr1 and Sp1 have been previously seen in the promoter of several genes. For example, it has been previously demonstrated that under quiescent conditions the promoters of PDGF-A chain (A) and β(1)-adrenergic receptor are occupied by Sp1, which regulates basal expression of these genes (20, 21). However, on stimulation, levels of Egr1 rise, allowing Egr1 to displace Sp1 from this region; thus, inducing the expression of PDGF-A as a response to the induction (21). Physical and functional cooperation between Sp1 and Egr1 has also been seen at IL-2Rβ promoter in T cells (22). Here, constitutive level of IL-2Rβ is maintained by Sp1, whereas on induction, Egr1 physically interacts with Sp1 to maximize transcription. Similarly, in our study, Egr1 expression in Jurkat cells resulted in the induction of pre-hsa-miR-106a followed by mature hsa-miR-106a. This finding seems to be in consonance with the observation that Sp1 is present in Jurkat cells at basal levels and may be responsible for basal transcription of this miRNA. Perturbation of this equilibrium by Egr1 would then up-regulate hsa-miR-106a; thus, negatively regulating IL-10. This notion was further supported by our observation that Egr1 stimulated hsa-miR-106a expression was negatively correlated with IL-10 levels. However, the mechanism by which Sp1 and Egr1 cooperate for regulation of hsa-miR-106a expression remains to be elucidated.

PHA and PMA have been known to induce vast array of genes by activating p38 (23), p42/44 ERK (24), and JNK MAP kinases (25). These 3 types of MAP kinases can be activated individually or simultaneously; thus, suggesting their independent signaling roles. Although PMA alone is known to induce the activation of Sp1 in T cells (4), both PMA and PHA have been shown to activate Egr1 in CEM cells (T cell line) (26). IL-10 has been previously shown to negatively regulate LPS-stimulated Egr1 expression in mouse macrophages (27). Thus, it would be interesting to study the effect IL-10 on Egr1 induced expression of hsa-miR-106a.

The regulatory loops and feedback mechanism for miRNA mediated posttranscriptional regulation of IL10 may be critical in maintaining immune homeostasis, and its dysregulation may lead to disease. It seems plausible that 3 regulatory mechanisms like transcriptional regulation, ARE mediated 3′ UTR regulation, and hsa-miR-106a mediated 3′ UTR regulation may critically regulate IL-10 expression. In fact, recently, it has been shown that inhibitory function of NKP46 ′CD49b ′CD3− NK cells recruited in the hepatic granulomas in experimental model of visceral leishmaniasis was correlated with high IL-10 production as a result of increased stability of IL10 mRNA (28). It is very likely that hsa-miR-106a mediated posttranscriptional con-
Western Blotting. Nuclear protein was prepared as described previously (33), and Western blotting was performed for Egr1, Sp1, and Lamin, as described in SI Materials and Methods.

Statistical Analysis. Results are given as mean of 3 independent experiments ± SEM. An independent 2-tailed Student’s t test was performed. Differences were considered statistically significant for P ≤ 0.05. For normalization of transfection efficiency, cells were cotransfected with vector pMIR-REPORT beta-Gal as indicated.

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Supporting Information

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SI Materials and Methods

**Plasmids.** Human *IL10* 3' UTR (AF148271, 1033 base pair, position +3831 to +4864) was amplified from genomic DNA, cloned in pMIR-REPORT vector (Ambion), and designated as pMIR-REPORT-IL10 3' UTR (intact).

To generate the microRNA target site deletion mutant, PCR based site-directed mutagenesis was performed by using pMIR-REPORT-IL10 3' UTR (intact) as template described (1,2) to delete 27 bases from -4451 to -4478 that constitute microRNA target recognition sequence. To generate clone of hsa-miR-106a promoter in pGL3 (basic) vector (Promega), 1-kb region of putative promoter from +1 to -1000 was amplified by PCR and cloned in the pGL3 vector.

**Cell Culture and Transfection.** A549 cells were grown in 75 cm² culture flask in Dulbecco’s modified Eagle’s medium (Sigma), containing 10% FBS, streptomycin (50 μg/mL), and penicillin (50 units/mL) at 37 °C in 5% CO₂. Raji cells, THP-1, and Jurkat cells were grown in RPMI (Sigma), containing 10% FBS, streptomycin (50 μg/mL), and penicillin (50 units/mL) at 37 °C in 5% CO₂.

For transfection, A549 cells were transfected with 800 ng of pMIR-REPORT or pMIR-REPORT-IL10 3' UTR (intact) or pMIR-REPORT-IL10 3' UTR (mutant) with or without end modified microRNA oligonucleotides (Dharmacon) by using Lipofectamine 2000 (Invitrogen). Reporter gene assay was performed 24 h after transfection by using luciferase assay kit (Promega). The cells were also cotransfected with 100 ng of pMIR-REPORT beta-Gal control for normalizing transfection efficiency. Beta galactosidase expression was assayed by using Beta-Gal assay kit (Promega).

Raji cells and Jurkat cells were transfected in 12-well plate by using Lipofectamine 2000 (Invitrogen); 1 million (10⁶) cells were exposed in Phospho Imager (Fujifilm FLA 2000IR) for 48-h exposure.

**EMSA.** Nuclear extracts were incubated with radio-labeled double stranded oligonucleotide probes for hsa-miR-106a potential promoter region (from -308 base pair to -330 base pair that was predicted to constitute potential transcription factor binding site). Protein-DNA complexes were separated from free DNA probe by electrophoresis through 5% nondenaturing acrylamide gels in 0.5 Tris borate ethylene diamine tetraacetic acid buffer (TBE). Super shift experiments used 1 μg each of anti-Egr1, anti-Sp1 antibody 20 min before adding the radiolabeled probe (Santa Cruz Biotechnology). Gels were dried, and protein complexes were visualized by autoradiography in a Phospho Imager (Fujifilm FLA 2000IR).

**Western Blotting.** Cytosolic and nuclear extracts were prepared from Jurkat cells (5 × 10⁶) as described (3). Proteins were separated by SDS/PAGE, and transferred to Hybond P membrane (Amersham Pharmacia Biotech). Egr1, Sp1, and Lamin were visualized using a 1:500 dilution of anti-Egr1 antibody, 1:1000 anti-Sp1, and 1:500 anti-Lamin (Santa Cruz Biotechnology). The detection was done by colorimetric assay using DAB (3,3'-Diaminobenzidine tetrahydro chloride) as a substrate.