Functional Analysis of Promoter Regions from Carp α-globin Genes in Cultured Fish Cells and Human Erythroleukemia Cells

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The 5′-upstream regions from the carp Cyprinus carpio α-globin gene family were fused to the bacterial reporter gene, the chloramphenicol acetyltransferase gene. The transcription role was examined in a transient expression assay after transfection into fish non-erythroid cell lines and human erythroleukemia cell lines. Our studies demonstrated that carp α-like globin genes have functional promoter regions. In the fish GF cell line, CAT activity was enhanced. There was no preferential expression of the carp α-globin promoter-CAT constructs in the human erythroleukemia cell lines, K562 and HEL, compared with that in fish non-erythroid cells.

The structure and expression of the human globin gene has been widely studied, and these studies have provided an interesting and important model of regulated gene expression during both ontogenic development and erythroid differentiation. Fish possess at least two major hemo-globin components similar to those found in higher vertebrates.1 In 1984, Takeshita et al.2 first cloned the cDNA of the α-globin gene from carp. By constructing a carp genomic library and screening the recombinant phages, the seven carp α-like globin genes were identified3 and designated as Nos. 1 to 7. The nucleotide sequences of these α-globin genes have been determined.3,4 Although the transcriptional control elements of mammals, yeast or drosophila for example, have been extensively characterized, little is known about these control regions in fish. So far only a few fish promoters have been studied. These include the promoter of the metallothionein B gene from rainbow trout,5 a β-actin gene from carp,6 antifreeze protein genes from cold-water fish,7 a α-globin gene from yellowtail,8 and a prolactin gene from chinook salmon.9 In the present study, the transcriptional control function and tissue-specific expression of the 5′-upstream regions from carp α-globin genes Nos. 3 to 7 were studied by a transient expression system.

Materials and Methods

Construction of Carp α-globin Promoter-CAT Recombinant Plasmid

The pRSVCAT10 was used as a base for inserting the carp α-globin promoter sequences. This plasmid contains the replication origin of the pUC19, an ampicillin resistance gene, SV40 splicing signal and SV40 polyadenylation signal. The CAT gene was placed downstream of the long terminal repeat (LTR) of Rous sarcoma virus control region. Before inserting carp α-globin promoter, the LTR was removed at the NruI and HindIII sites. The upstream region of the No. 3 carp α-globin gene was cut from the HindIII site at -627 base pairs (bp) (all sites are defined relative to the cap site), to the MboII site at +21 bp; the upstream sequence of No. 4 was cut from the AluI site at about -879 bp to the AluI site at +21 bp; Nos. 5 and 7 was cut from the HindIII site at -248 bp to the MboII site at +21 bp; No. 6 from the HindIII site at -250 bp to the MboII site at +21 pb. All the upstream fragments were blunted and subcloned at the HinclI site of plasmid pUC119, then the α-globin upstream fragments were cut out of the pUC119 polylinker with Smal and HindIII, ligated to the pRSVCAT in which the LTR control region was removed (Fig. 1). The resulting hybrid plasmids were designated as P-No. 3-CAT, P-No. 4-CAT, P-No. 5-CAT, P-No. 6-CAT, and P-No. 7-CAT.
Fig. 1. Structure of carp α-globin promoter-CAT genes.

6-CAT, P-No. 7-CAT respectively.

Cell Culture

The following established cell lines were used: TO-2 established from the ovary of tilapia hybrid, Tilapia mossambica × T. nilotica;11) GF(FI) from the fin cells of the colored carp Cyprinus carpio.12) TO-2 and GF were grown in Leiboviz's L-15 medium (L-15) supplemented with 10% fetal bovine serum, penicillin (250 U/ml), streptomycin (250 µg/ml). The cells were incubated at 28°C and subcultured at 5 day intervals with 0.1% trypsin in EDTA/PBS(−) solution at room temperature. All sera and culture media were purchased from Flow Laboratories Inc., Maryland, U.S.A.

K562 cells13) were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in a humidified 5% CO₂ environment and subcultured at five-day intervals with 0.25% trypsin in 0.02% EDTA/PBS(−) solution at room temperature. All sera and culture media were purchased from Flow Laboratories Inc., Maryland, U.S.A.

Transfection

Foreign DNA was introduced into fish cells and human erythroleukemia cells by calcium phosphate precipitation treatment. On the day before transfection, healthy growing cells were planted at a density of 5 × 10⁵ cells/10 cm well. Calcium phosphate DNA precipitates were prepared by the method of Ausubel et al.15) with minor modifications. The precipitate mixture was incubated for 20 min at room temperature, transferred to the cells, and incubated for 8 h at 28°C and 37°C for fish and human cells respectively. After the medium was removed the cells were osmotically shocked with 15% glycerol for 1 min and washed 3 times with serum-free medium. The transfected fish and human cells were allowed to incubate for 48 h at 28°C and 37°C respectively.

CAT Assays

CAT assays were performed essentially as described previously.8) Two hundred µg cell extract protein were used for each assay. Prior to incubation with substrate, the cell extracts were heated to 65°C for 10 min to destroy any interfering activity. The CAT assay was performed at 37°C. Thin-layer chromatography plates were analyzed using an AMBIS radioanalytic imaging system (AMBIS system Inc., San Diego, California, USA).

Results

Introduction of Exogenous DNA into Cultured Fish Cells

For the effective introduction of exogenous DNA into cultured fish cells, the plasmids used for transfection were supercoiled and purified twice by equilibrium centrifugation. Comparison of CAT activity after transfection with different methods revealed that the calcium phosphate method15) was most effective. It was necessary to check the precipitate formation before mixing with the desired DNA. If less DNA is to be used, the DEAE-dextran/glycerol method is preferable. Before DEAE-dextran treatment, adherent cells
Fig. 2. CAT activity of GF cell extracts.
The cells were transfected with plasmids pRSVCAT in which the RSV-LTR promoter region was deleted (A); pRSVCAT (B); P-No. 3-CAT (C); P-No. 4-CAT (D); P-No. 5-CAT (E); P-No. 6-CAT (F) and P-No. 7-CAT (G).
Abbreviations: CM, unacetylated chloramphenicol; CM-AC₁, 1-acetylchloramphenicol; CM-AC₃, 3-acetylchloramphenicol.

Fig. 3. CAT activity of GF cell extracts.
The cells were transfected with A: pRSVCAT in which the RSV-LTR was deleted, B: P-No. 3-CAT, C: P-No. 4-CAT, D: P-No. 5-CAT, E: P-No. 6-CAT, F: P-No. 7-CAT.

Transient Expression of CAT Gene under the Control of Carp α-globin Upstream Sequence in Cultured Fish Cells

The upstream sequences from carp α-globin genes Nos. 3 to 7 were isolated to control the expression of the CAT gene, and these fragments containing a complete TATA box were located 84 nucleotides upstream of the ATG initiation codon. No CCAAT box could be found in a position about 40 nucleotides upstream from the TATA box. However, this region was very similar to that of yellowtail, suggesting that the fish α-globin gene has a different component that replaces the role of the CCAAT box. The cap site is located 51 nucleotides upstream from the ATG initiation codon.¹³,⁴

To assess the activity of each globin gene promoter in fish cells, we prepared plasmid constructs of each promoter to a reporter CAT gene (Fig. 1). These constructs were transfected into cultured fish cell lines GF and TO-2 for an assay of CAT activity. The results shown in Figs. 3 and 4 are the average data for the three experiments. The
Table 1. Two-way classification of CAT activities directed by carp α-globin promoters in four cell lines

<table>
<thead>
<tr>
<th>Promoter</th>
<th>GF</th>
<th>TO-2</th>
<th>K562</th>
<th>HEL</th>
<th>X_i</th>
<th>Σ X_i</th>
<th>Σ X_i^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10.6</td>
<td>2.3</td>
<td>1.9</td>
<td>1.2</td>
<td>3.8</td>
<td>15.1</td>
<td>122.74</td>
</tr>
<tr>
<td>4</td>
<td>5.6</td>
<td>0.6</td>
<td>1.4</td>
<td>0.9</td>
<td>2.1</td>
<td>8.5</td>
<td>34.48</td>
</tr>
<tr>
<td>5</td>
<td>3.5</td>
<td>1.0</td>
<td>1.7</td>
<td>1.1</td>
<td>1.8</td>
<td>7.3</td>
<td>17.35</td>
</tr>
<tr>
<td>6</td>
<td>3.8</td>
<td>1.3</td>
<td>1.7</td>
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<td>2.0</td>
<td>7.8</td>
<td>20.02</td>
</tr>
<tr>
<td>7</td>
<td>3.9</td>
<td>0.5</td>
<td>1.3</td>
<td>1.1</td>
<td>1.7</td>
<td>6.8</td>
<td>18.36</td>
</tr>
</tbody>
</table>

X_i: 5.5, X_i: 27.4, X_i: 185.61

GF and TO-2 cells transfected by carp α-globin promoter sequences-CAT hybrid plasmids have obvious CAT activity, compared with the negative control. This suggests that the promoter sequences from the carp α-globin gene possess the function of a promoter in vitro.

Transient Expression of CAT Gene under the Control of Carp α-globin Promoter Sequences in Human Erythroleukemia Cells

Human erythroleukemia cell lines that produce variable amounts of globin were obtained. The K562 cell line was derived in 1975 and is the one most extensively studied. The HEL line is another human erythroleukemia cell line. These cells contain genes for all the hemoglobins. They produce all globin chains except for adult β-globin.

The carp α-globin promoter-CAT constructs, which consist of the 5'-upstream sequences of the carp α-globin gene, were transfected into these cell lines. The CAT expression in these cells is shown in Fig. 5. The data are the average of three experiments.

Biological Statistics of Activities between Promoters and Cell Lines

Two-way classification was applied to analyze the CAT activities directed by carp α-globin promoters No. 3 to 7 in GF, TO-2, K562, and HEL cell lines. The related values are shown in Table 1 and Table 2. The result indicated that the mean of CAT activities directed by carp α-globin promoters No. 3 to 7 were not significantly different from each other. On the other hand, in the GF cell line the mean of CAT activities directed by carp α-globin promoters was significantly stronger than in TO-2, K562, and HEL cell lines.

Transient Expression of CAT Gene under the Control of RSV-LTR in Fish Cell Lines and Human Erythroleukemia Cell Lines

A variety of viral promoter and enhancer sequences have been identified as active in mammalian cell lines, and similar sequences have been proved active in tissue cultured fish cells. In order to compare the activity of carp α-globin promoter sequences with the strong promoter RSV-LTR in the same cell lines, the pRSVCAT

Table 2. Variance analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>19</td>
<td>107.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell line</td>
<td>3</td>
<td>69.27</td>
<td>23.09</td>
<td>9.74*</td>
</tr>
<tr>
<td>Promoter</td>
<td>4</td>
<td>9.36</td>
<td>2.34</td>
<td>0.98</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>28.43</td>
<td>2.37</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.01
GF > TO-2 > K562 > HEL
a > b p < 0.01.

Fig. 5. CAT activity in K562 and HEL cell extracts.

The cells were transfected with A, B: pRSVCAT in which the RSV-LTR was deleted, C, D: P-No. 3-CAT, E, F: P-No. 4-CAT, G, H: P-No. 5-CAT, I, J: P-No. 6-CAT, K, L: P-No. 7-CAT.
Functional Analysis of Carp α-globin Promoter

Discussion

The regulation of human globin gene expression has been widely studied. Until now, there has been no information concerning the regulation of fish globin gene expression except for our previous work with the carp α-globin genomic gene23) and the promoter region from yellowtail α-globin gene.8) In the present study, the promoter sequences isolated from the carp α-globin gene family were linked to a reporter gene, the chloramphenicol acetyltransferase gene. These constructs were introduced into fish non-erythrocytic cells and human erythroleukemia cells. The expression of the CAT gene in these cells was analyzed indicating that the promoter regions from carp α-globin gene fulfill their function in these cells. In GF cells the CAT activity directed by the carp α-globin promoter sequences was significantly higher than in TO-2, K562, and HEL cells. In the human erythroleukemia cell lines, K562 and HEL, there was no preferential expression of the CAT gene directed by carp α-globin promoters observed. This may be because a fish gene promoter may be not subject to the same stringency of control in heterologous human cells.

In this study, the 5′-upstream regions from −627 to +21 bp for the No. 3 carp α-globin gene, −879 to +21 bp for No. 4, −248 to +21 bp for the Nos. 5 and 7, and −250 to +21 bp for No. 6 gave an intermediate strength of expression as compared with the heterologous SV40 early promoter4) and RSV-LTR in cultured fish cells. These data show that the 5′-upstream regions from carp α-globin gene can be used as a homologous promoter for the expression of foreign genes in cultured fish cells.

Acknowledgments

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References

13) C. B. Lovato and R. B. Lovato: Human chronic myelogenous leukemia cell line with positive philadelphia chromosome.


