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# Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes

**Research Article**

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## Summary

**AAV vectors have become a practical choice for gene transfer into a variety of tissues *in vivo*. For the purpose of supplemental gene therapy, liver and skeletal muscles are the major targets of transduction. Recently, vector serotypes and their tissue specificity is an important issue for successful gene transfer. Although the potential utility of each serotype has been announced, a systematic comparison has not been fully made. In addition, the choice of a suitable promoter is also critical. In this study, we investigated the level of the transgene product by comparing different serotypes as well as promoters. We utilized murine erythropoietin (*mEpo*)-expressing or *LacZ*-expressing AAV vectors and transferred them into immune-competent mice. For the muscle-directed expression, serotype 1 showed the highest Epo expression followed by 5, 2, 3 and 4. As for the promoter analysis in the liver, the CAG promoter achieved the highest Epo concentration, followed by CMV, PGK, and EF-1α. Further comparison in the liver with different serotypes utilizing the CAG promoter revealed that serotype 5 showed the highest level of expression. These results will aid in the design of an optimal AAV vector structure, especially for the systemic delivery of transgene products.**

## I. Introduction

AAV vectors are non-pathogenic, can transduce a wide range of tissues, and hold promise as a choice for efficient gene transfer toward a variety of applications including therapeutic approaches in humans. Among a panel of candidate diseases, hemophilia B is considered one of the best-suited models, and results of a human trial has already been documented (Kay et al., 2000). Although the potential usefulness of the AAV vector has been indicated, there is a need to improve the level of transgene expression, especially for the approaches which require the release of transgene products into the systemic circulation. For this purpose, muscle and liver are the preferred targets of transgene expression. Therefore, we made several improvements to optimize the expression profiles in these tissues. One approach is to find a choice of promoters and enhancer elements. The other issue is the use of appropriate serotypes. As for the human concern, AAV consists of 5 naturally occurring serotypes and there are possibilities that other serotype-based vectors possess higher utilities in certain applications than the 'classic' type 2. Recent progress has made it possible to develop these serotypes as vectors (Muramatsu et al., 1996; Chiorini et al., 1997; Chiorini et al., 1999; Xiao et al.,

1999). Recently, two novel serotypes were added to the list (Gao et al., 2002). At the same time, differences in tropism have gradually been substantiated by the discovery of molecules involved in the attachment to and entry of cells (Kaludov et al., 2001; Walters et al., 2001; Pasquale et al., 2003). Although the utilities of each serotype-based vector were suggested by the developers, there are few studies to compare usefulness on the same platform. For this purpose, we designed vectors encoding murine erythropoietin with various promoters and available serotypes and quantitated the level of transgene expression and biological consequences following injection.

## II. Materials and methods

### A. Cell lines and construction of the plasmids

HEK293 cells were maintained as described previously (Ogasawara et al., 1999). Plasmid pW1, which harbors the bacterial LacZ gene under the control of the CMV promoter (**Figure 1**), was utilized as a marker for transgene expression. Common molecular biology reagents e.g. restriction enzymes, were purchased through Takara Bio Inc., (Ohtsu, Japan). Plasmid p3.3.1c, which harbors a CMV promoter followed by a multiple cloning site and a poly A sequence, was a gift from Avigen, Inc. Plasmids encoding various promoters for comparison in this study were constructed by replacing the sequences of the CMV promoter of p3.3.1c with the corresponding sequence; Plasmid p3CAG was constructed by removing the Spe I - Cla I fragment from p3.3.1c, filled-in and ligated with a 1.6 kb EcoR I – Hind III fragment of pCAGmcs. Plasmid p3GCAG containing a Gst enhancer was constructed with the Cla I – Hind III fragment of pGST-E-CAG (a gift from Dr. T. Naruse, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) into a blunted Cla I- Spe I backbone of p3.3.1c. Plasmid p3EF was constructed in the same way in that the Spe I - Cla I fragment of p3.3.1 was replaced by the Hind III-EcoR I fragment of pEF-BOS (a gift from Dr. Stuart H. Orkin, Boston, MA). p3PGK was also generated by replacing Mlu I-BstB I sequences with the EcoR I-Hind III fragment of pUC/PGK (Lim et al., 1987) provided by Dr. D Williams (Indiana University, Indianapolis, Indiana) by blunt-end ligation. For the p3PGKG plasmid, Mlu I-Sac II sequences were replaced with PGK sequences.

### B. Murine erythropoietin sequence

Murine erythropoietin cDNA was constructed as follows. Total RNA was extracted from kidneys of an 8 week-old C57BL/6 mouse with Isogen (Nippongene, Tokyo, Japan), and reverse transcribed with Superscript II (Invitrogen Corp., Carlsbad, CA) using an oligo (dT) primer and the manufacturer's instruction. Then PCR was performed using a cDNA library as a template. Primers for PCR were MEPO5 : 5'-CGG AAT TCA TGG GGG TGC CCG AAC GTC CCA-3', MEPO3-2 : 5'-TAG GAT CCT GGC AGC AGC ATG TCA CCT GTC-3', and the conditions were 3min at 92 °C, followed by 25 cycles of 98 °C for 30 sec, 58°C for 1min, 72°C for 1min using pfu polymerase and the standard buffer (Stratagene, La Jolla, CA). Following the reactions, the product was digested with EcoR I and BamH I and inserted into the corresponding sites of pBluescript II sk (+). Murine Epo sequences was inserted into the plasmids containing the promoter sequences. Finally, the Not I fragments of the expression cassettes were subcloned into the Not I sites of the ITR-containing plasmid.

### C. AAV vector production system

We used production systems for AAV serotypes 1-5 based on the following materials and information. The AAV vector production system for serotype 2 was obtained from Avigen Inc. The production system for serotype 3 was already developed (Muramatsu et al., 1996), and the system for serotype 4 and 5 production (Chiorini et al., 1997; Chiorini et al., 1999) was a gift from Dr. Chiorini (NIDCR, NIH). For the serotype 1-derived vector production system, we modified the helper plasmid of serotype 2 by replacing the capsid sequence for serotype 1. Wild type AAV serotype 1 virus was obtained from ATCC (Manassass, VA). Following the extraction of viral DNA by heat denaturation at 100 °C for 5 minutes, the sequences corresponding to the serotype 1 capsid were amplified by standard PCR, utilizing the following primers for sense and

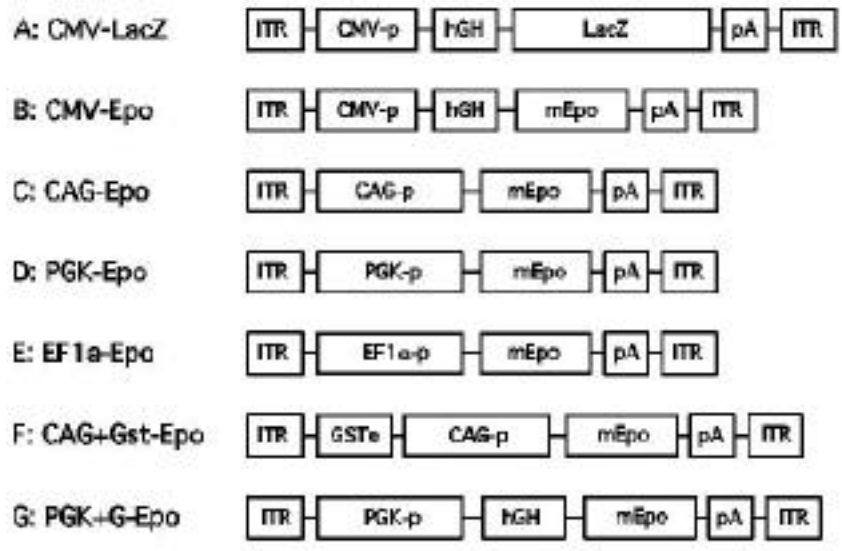



**Figure 1**. The structures of the helper plasmids and the vectors. The promoters used in this study were CAG, CMV, EF-1α, and PGK and tested for the capability of expression in the liver. As for the LacZ expression, only the CMV promoter was used due to the size restriction of the transgene cassette for AAV vectors.

antisense; 5'- agc ttt gtT TAA ACC AGG TAT GGC TGC CGA TG and 3'- agc ttt gtt tAA ACG AAT CAA CCG GTT TAT TGA T, respectively using pfu polymerase and the standard buffer (Stratagene, La Jolla, CA). The 2.3kb PCR fragment was digested by Pme I, and ligated into the Swa I-SnaB I sites of the HLP19 plasmid. As for the vector production, we utilized the transfection of triple plasmids into HEK293 cells as described previously (Matsushita et al., 1998).

### D. Vector purification

To avoid potential interference of CsCl with the specific serotype-derived vectors (Auricchio et al., 2001), we utilized Iodixanol as a separation medium based on the reports (Hermens et al., 1999; Zolotukhin et al., 1999). In order to improve the purity, we utilized high salt conditions during the separation period of ultracentrifugation (Arella et al., 1990). The separation medium consists of 2M NaCl, 33% Iodixanol and 50mM Hepes. The separation was performed twice for 3hours, with the use of a vertical rotor VTI50 (Beckman, CA). The fractions corresponding to the full capsid were recovered, dialyzed against 50mM Hepes-150mM NaCl, and finally concentrated to around 1 x $10^{10}$ vg/μl. The titers of the vector stocks were determined by dot blot analysis utilizing BAS 1500. The integrity of the vectors was tested by infecting 293 cells with LacZ-encoding vectors, and the results were similar to those obtained with the CsCl purified vectors (data not shown).

### E. Animal experiments

All of the animal experiments were performed according to the guidelines of the Jichi Medical School. C57BL/6J mice were purchased from CLEA Japan, Inc., and were subjected to vector injection at 6 weeks of age. For the intramuscular injection, 6 x $10^{10}$ genome copies of each serotype vector, dissolved in 50 μl of saline, was injected into bilateral tibialis anterior muscle. For the portal vein injection, 1 x $10^{11}$ genome copies of each serotype vector, dissolved in 400 μl of saline, was injected under laparotomy. For the promoter analysis, AAV2-based vectors encoding Epo were injected at 1 x $10^{10}$ genome copies to analyze the difference in more detail. Whole blood was collected from these animals every 2 weeks and analyzed for complete blood counts including hemoglobin content and plasma Epo concentrations. Complete blood counts were performed using a PC-608 particle counter (Erma, Tokyo, Japan). Plasma Epo concentrations were quantified with an ELISA kit purchased from Roche Diagnostics Inc. (Mannheim, Germany). Error bars indicate standard deviations throughout the study and the statistical significances were analyzed by Welch's t-test.

### F. Tissue preparation and X-Gal staining and immunohistochemistry

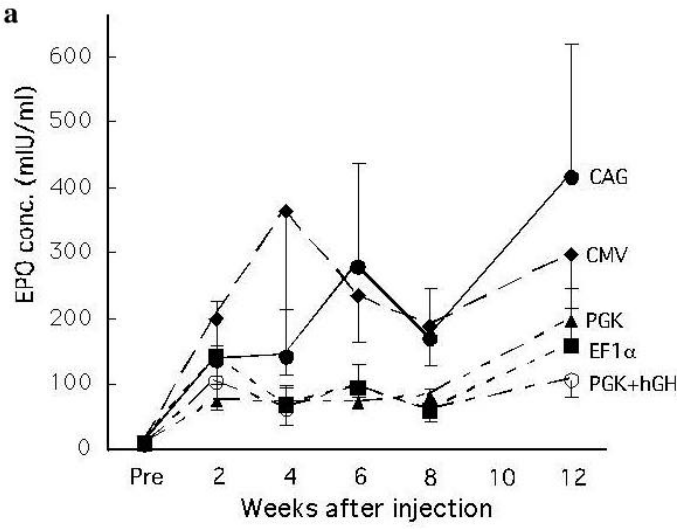
Following the observation period, the mice were sacrificed, and the target organs were recovered. These sections were frozen fresh, and sliced with the microstat. The sections were stained with X-Gal. At the same time, these sections were collected, and the β-galactosidase activity was measured using a β-Gal Assay kit (Pierce, Redford, Illinois), then adjusted according to the protein content of the sample determined with BCA kit (Pierce, Redford, Illinois).

The immunohistochemistry was performed with these sections. A goat polyclonal antibody against human Epo (sc-1310, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was utilized at a concentration of 10 μg/ml. Following a reaction with biotinylated anti-goat IgG (Vector laboratories, Burlingame, CA), horseradish peroxidase-conjugated streptavidin (Vector laboratories, Burlingame, CA), was used following the manufacturer's instructions. The overall reactions were visualized using the DAB-$H_2O_2$ system.

## III. Results

### A. Effect of promoter selection on liver-directed transduction

In our series of experiments to compare the promoter activity, CAG and CMV promoters showed the highest expression of Epo. For the first 4 weeks, CMV showed the strongest expression; thereafter, CAG showed the highest level of Epo expression. No differences were observed between these groups. EF-1α and PGK promoters showed significantly lower levels of Epo expression relative to the former promoters (**Figure 2a**). As for the effects upon red blood cell counts, no significant differences were observed among the groups with different promoters (**Figure 2b**). In order to analyze the difference in more detail, the promoter activities were tested at the lower dose of the vectors.



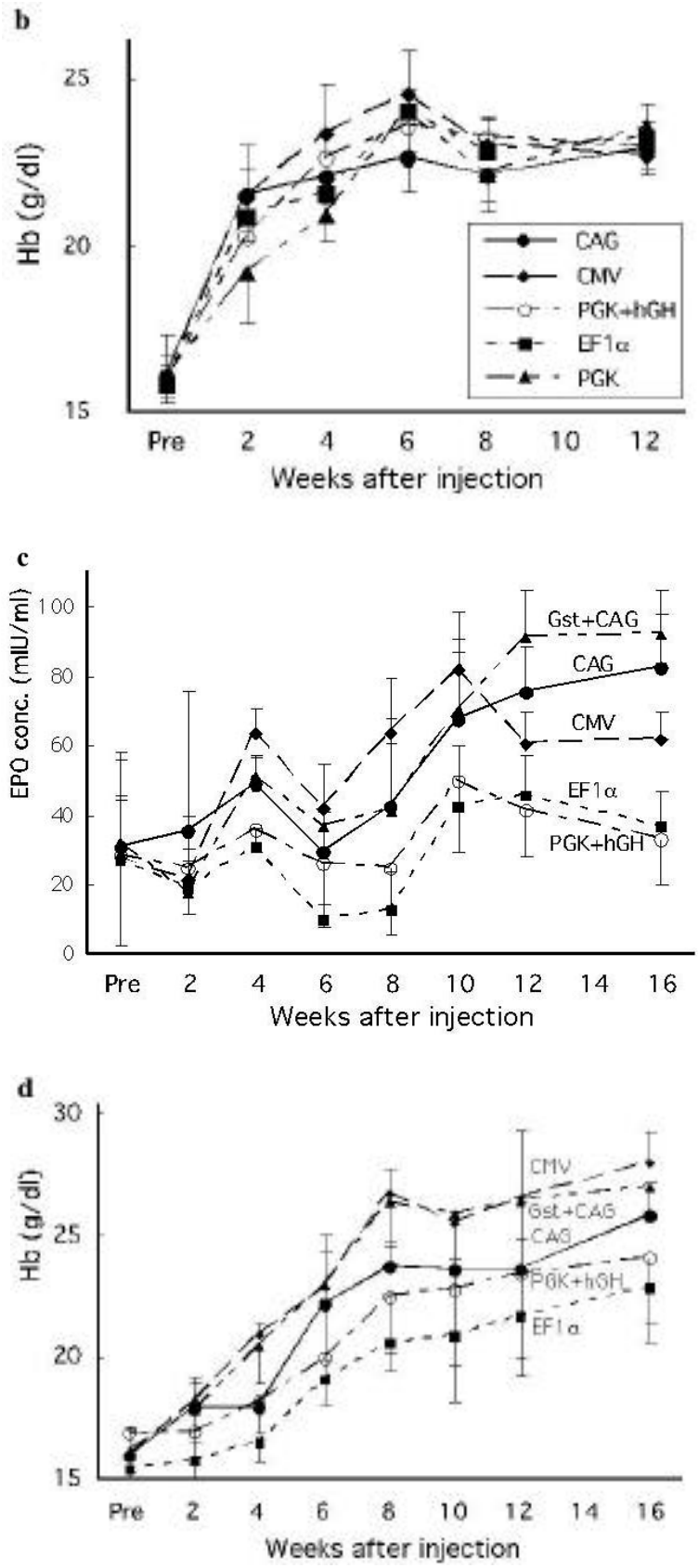



**Figure 2**. Expression profiles for Epo according to the choice of the promoter following intraportal injection of the vectors. The vectors utilized various promoters to drive Epo with the serotype 2 capsid. A higher dose ($1 \times 10^{11}$ vector genomes per body) was used in the upper panels showing **a**) plasma Epo concentration, **b**) Hemoglobin content of the blood. **c)** and **d**) corresponds to these profiles with the lower dose ($1 \times 10^{10}$ vector genomes per body). CMV and CAG promoters showed higher expression levels than the rest of the promoters..

As shown in **Figure 2c**, CAG promoters showed the highest expression, followed by CMV, EF-1α and PGK promoters. No significant differences were observed among the data with CAG, CMV and Gst+CAG promoters, whereas the results with EF-1α and PGK promoters were significantly lower than the rest. At this vector dose, plasma hemoglobin content showed differences among groups, mostly consistant with the degree of Epo expression (**Figure 2d**). Statistic analysis revealed that the results with EF-1α were significantly lower than the rest of the groups.

## B. Serotype-related difference in expression profiles in the liver

For the liver-directed transgene expression, we compared serotype-based vectors encoding LacZ. Serotype 5-based vector showed the highest number of cells as assessed by the X-Gal staining of tissue sections. A typical micrograph of X-Gal staining in the case of AAV-5 is shown in **Figure 3a**. The overall results with the X-Gal-positive cells *in vivo* are summarized in **Figure 3b**. The majority of the X-Gal-positive cells were hepatocytes, regardless of the serotype.

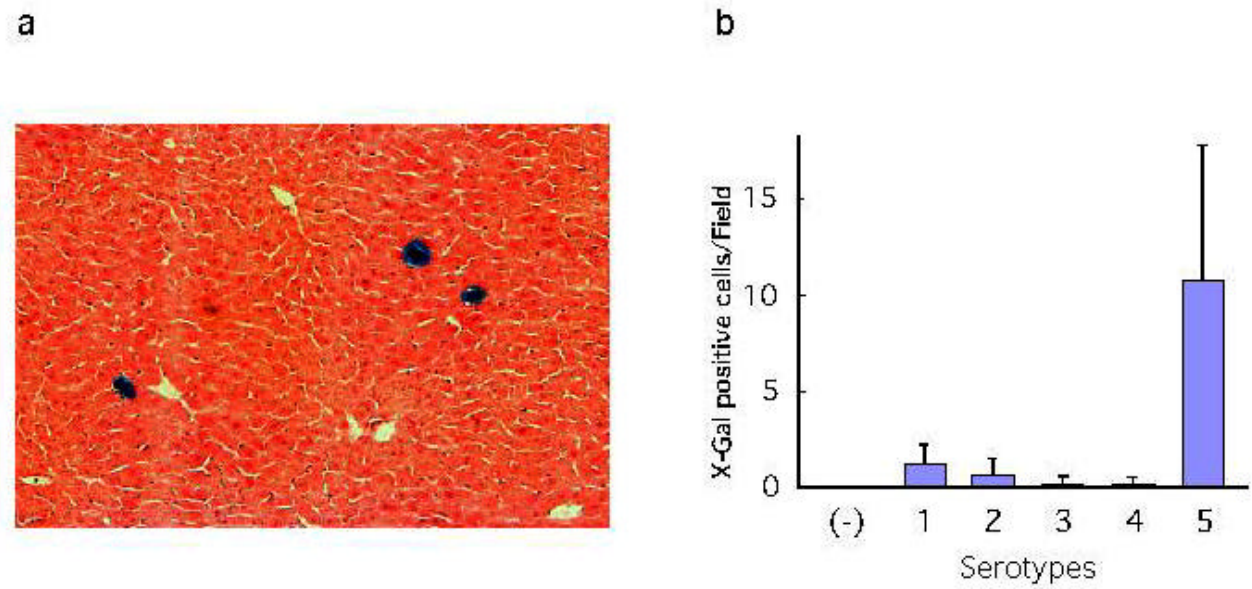
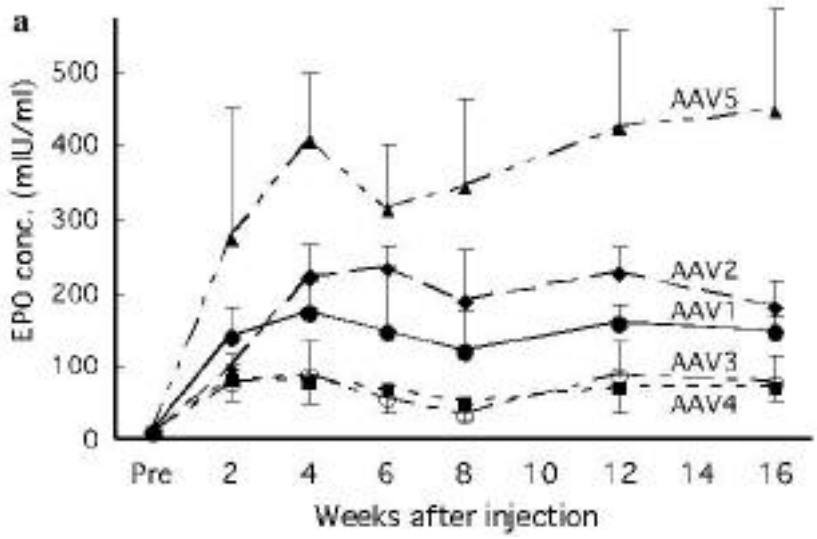



**Figure 3.** Histological analysis of liver tissue following intraportal injection (1 x $10^{11}$ vector genomes) of the serotype-derived vectors. LacZ-encoding vectors with CMV promoter utilizing serotype-derived capsid were injected, and the mice were sacrificed 2 weeks after the injection. The liver was sliced and X-Gal staining was performed. **a**) a representative photograph following injection with serotype 5-derived vectors. Original magnification was x 4. The slides were counterstained with eosin. **b**) the number of X-Gal-positive cells within a field under low magnification are shown. At least 5 different fields were observed, and the numbers were statistically analyzed. Serotype 5- based vectors showed a higher transduction rate than the rest of the groups ($p < 0.05$).



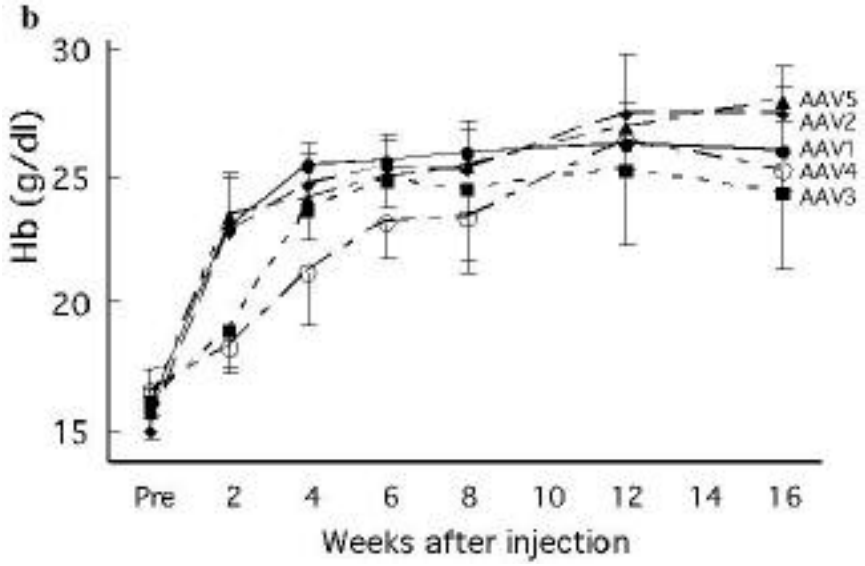



**Figure 4.** Expression profiles of Epo following intraportal vector injection. Serotype-derived vectors using the CAG promoter were produced, and the concentrations of Epo were compared. **a**) among the serotypes, AAV-5 showed the highest Epo concentration. **b**) the difference in hemoglobin content and other red blood cell parameters tended to be similar.

In experiments with Epo genes, the results were almost comparable to that with LacZ; AAV-5 showed the highest expression, followed by AAV-1 and 2. In the cases of AAV-3 and 4, the expression was the lowest (**Figure 4a**). All animals became polycythemic regardless of the degree of Epo expression (**Figure 4b**).

### C. Serotype-related difference in expression profiles in the muscle

In the experiments with LacZ-encoding vectors, AAV-1 and 5 showed the highest levels among all the serotypes. The rest showed a similar degree of expression by X-Gal staining (**Figure 5a-e**). The activity of β-galactosidase reflected the color of the X-Gal staining (**Figure 5f**). In terms of Epo expression, AAV-1 had the highest level, followed by 5, 4, 3 and 2, respectively (**Figure 6a**). Every group was significantly different from others. A prompt increase in the blood cell count was observed in every serotype, without significant difference among serotypes in this regard, as in the case of liver mediated gene transfer (**Figure 6b**).

### D. Histological examination of the tissues

Specific staining in the tibialis anterior muscle was observed in animals transduced with vectors encoding Epo (**Figure 7a**). A reaction without the use of primary antibody was performed as a negative control and is shown in **Figure 7b.**

## IV. Discussion

In this study, we compared the level of transgene expression using serotype-based vectors as well as promoters. Theoretically, the use of a capsid relates to the gene transfer efficiency, and the choice of the promoter / enhancer element affects steps for expression of the transferred gene. In order to search for the optimum combination, it is ideal to use a transgene whose product can be quantitated, and the biological consequences of which can be estimated without triggering immune reactions. For this purpose, we chose murine erythropoietin cDNA as a transgene to focus on the differences within the vector structure *per se*. Our results indicate that for the muscle-mediated expression, AAV-1 has unparalleled capacity among the serotypes tested. This result is consistent with the data reported by other groups (Chao et al., 2000; Rabinowitz et al., 2002), using different transgenes. As for the liver, the highest expression was attained by the use of AAV5-based vectors, which was also reported recently using other transgenes (Mingozzi et al., 2002). The molecular basis of these differences is still mostly unknown, despite steady progress in finding the steps in the transduction route (Walters et al., 2001).

As for the choice of the promoters for liver, CAG and CMV promoters were superior to the rest in experiments with various vector titers. No significant differences between the results using the CAG and CMV promoters were observed at the vector doses, although CAG tended to be higher at most of the data points. For the liver-directed gene therapy, there seems to be a discrepancy among the reports. The CBA promoter was reported superior to the CMV promoter in liver-mediated gene transfer (Xiao et al., 1998; Xu et al., 2001). The CBA promoter used in these studies is essentially the same as the CAG promoter used in this study (Niwa et al., 1991). The reasons for these differences are not clear, but may relate to the difference in transgene, strain of mouse, or technical details concerning the vector injection. In the present study, we utilized an immunocompetent strain of mouse and as the CAG promoter is about 2kb in length, the application of this promoter might be relatively limited in the case of AAV vector-mediated gene transfer. Adding the Gst enhancer sequence to CAG promoter was not

beneficial in this system. In addition, appending the intron sequences from human growth hormone did not affect the activity of PGK promoter (**Figure 2a**). It is interesting that in liver-mediated gene transfer, approximately 5-fold difference was observed in expression in most cases by the 10-fold difference in vector dose indicating non-linear response (**Figures 2a** and **2c**). In our series of experiments, we focused on the strongest promoter to

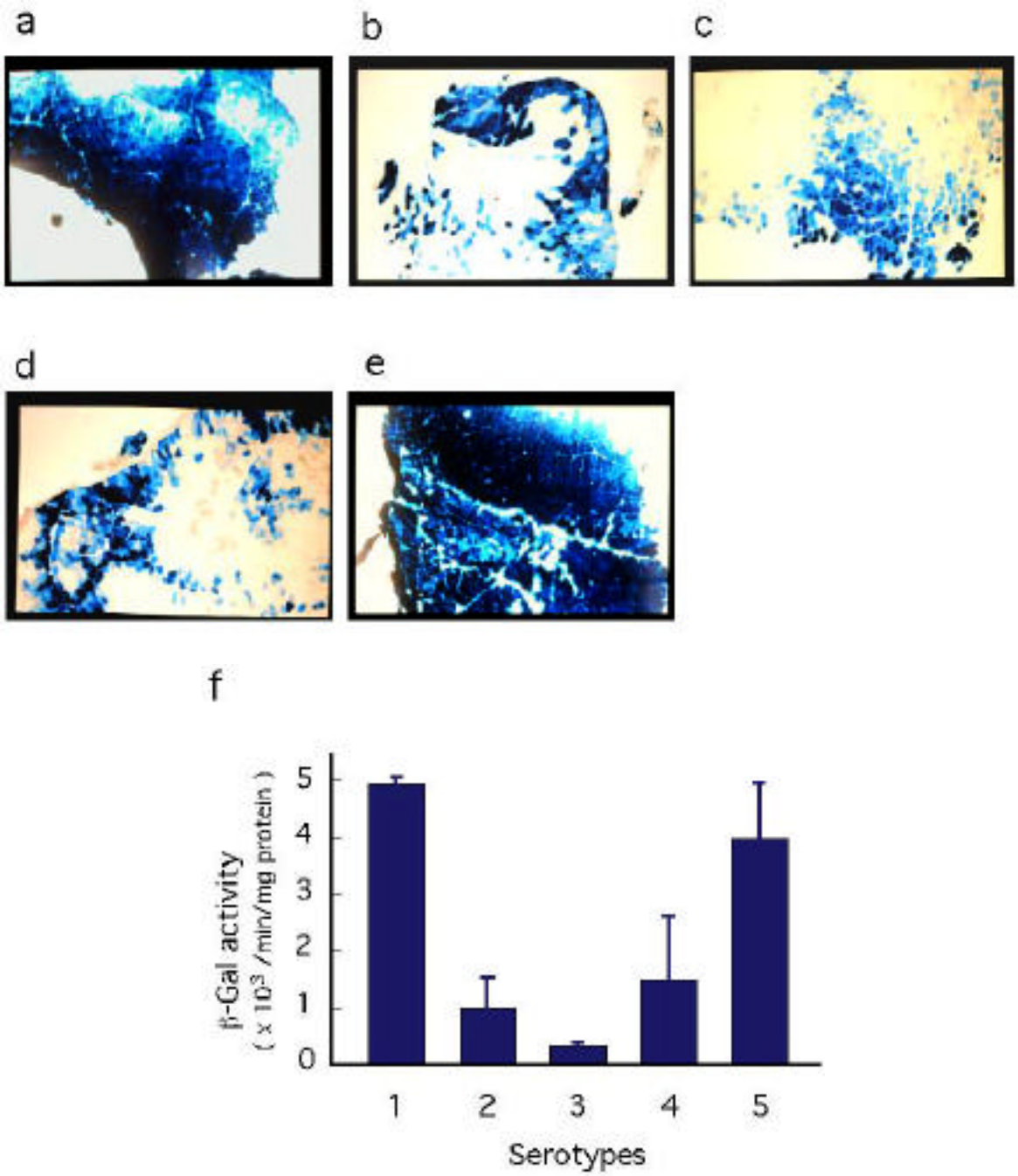



**Figure 5**. Expression of LacZ following intramuscular injection of the serotype-derived vectors. Each serotype-derived vector with the CMV promoter (3 x $10^{10}$ vector genomes per muscle) was injected into the tibialis anterior muscle of mice. Two weeks following the injection, mice were sacrificed and the muscle was histologically analyzed. X-Gal staining was performed, and the best image was chosen. **a)-e)** corresponds to the serotypes 1-5. **f)** The activity of β-Galactosidase was analyzed using the sections of muscle. Ten slices of the muscle specimen (10 μm of thickness) were collected into tube and the activity of the enzyme was determined based on the ONPG assay (Invitrogen Corp., Carlsbad, CA). The protein content was also determined by the BCA method (Pierce, Rockford, IL) to adjust the enzyme activity.

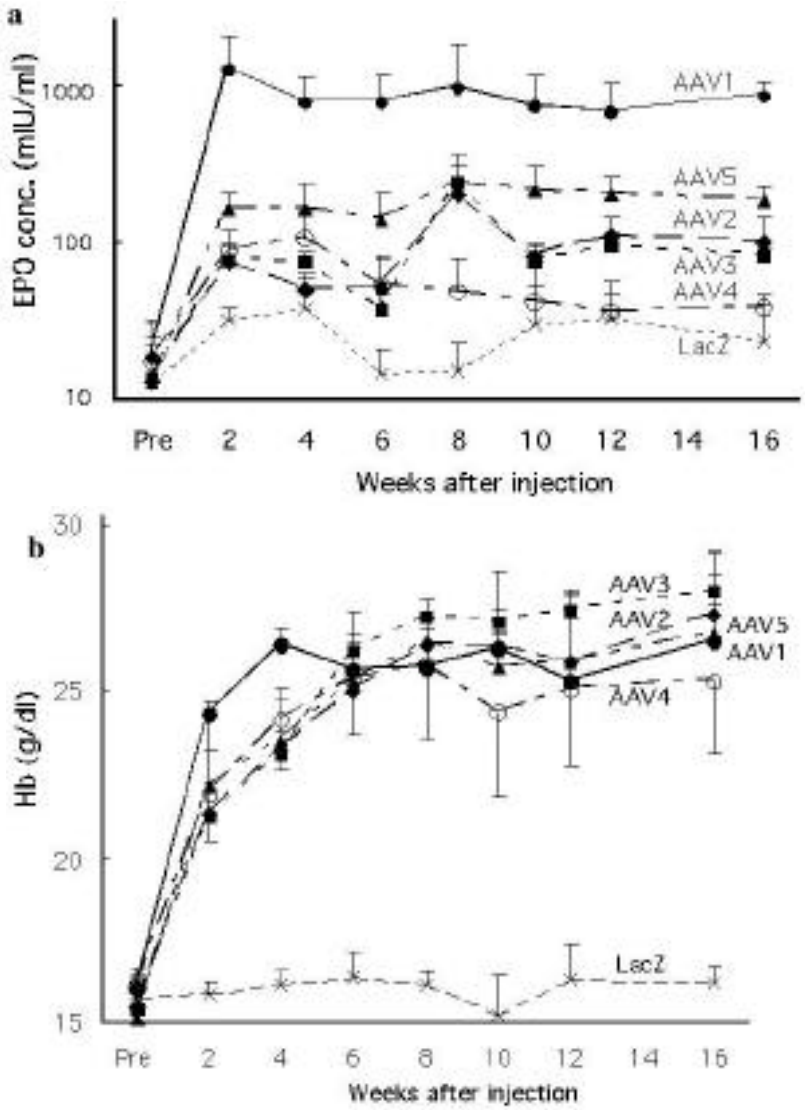



**Figure 6**. Muscle-directed Epo expression using serotype-derived vectors under the control of the CMV promoter. Bilateral gastrocunemius muscles were transduced by injecting 3 x $10^{10}$ vector genomes per muscle. **a**) Serum Epo concentrations were monitored. Animals injected with serotype 1- derived vectors showed the highest concentration throughout the observation period. **b)** Hemoglobin content of the blood showed a significant increase in every serotype.

drive the transgene. In clinical applications, tissue-specific promoters would be preferred for safety, to make sure that the expression of the transgene is limited to the target tissues. However, tissue-specific promoters are generally much weaker than the 'ubiquitous' promoters used in this study. Therefore, the tissue-specific promoters will become a practical choice if a more efficient method to transfer genes into the target tissue is developed. In the case of promoters for muscle, the CMV promoter seems to be the best match with a long history of expression of therapeutic genes (Song et al., 1998). The situation is similar to the liver in that several tissue-specific promoters are expected for future use especially the MCK promoter (Hauser et al., 2000). Approaches to develop synthetic promoters are also promising (Li et al., 1999).

In our series of experiments, there was a discrepancy in the results with LacZ and erythropoietin. In the experiments for muscle transduction, results with LacZ showed a comparable expression using AAV-1 and 5, whereas in the erythropoietin experiments, results with AAV-1 showed much higher expression. In this case, the amount of X-Gal seems extremely high, so the β-galactosidase activity within the myocyte may reach a plateau level. As erythropoietin is likely to be released efficiently from the muscle, its serum concentration reflect the production rate within muscle tissue. Immunohistochemical analysis supports persistent expression from transduced tissue (**Figure 7**). In our results for liver-directed gene transfer, the number of X-Gal-positive cells is relatively small. On the other hand,

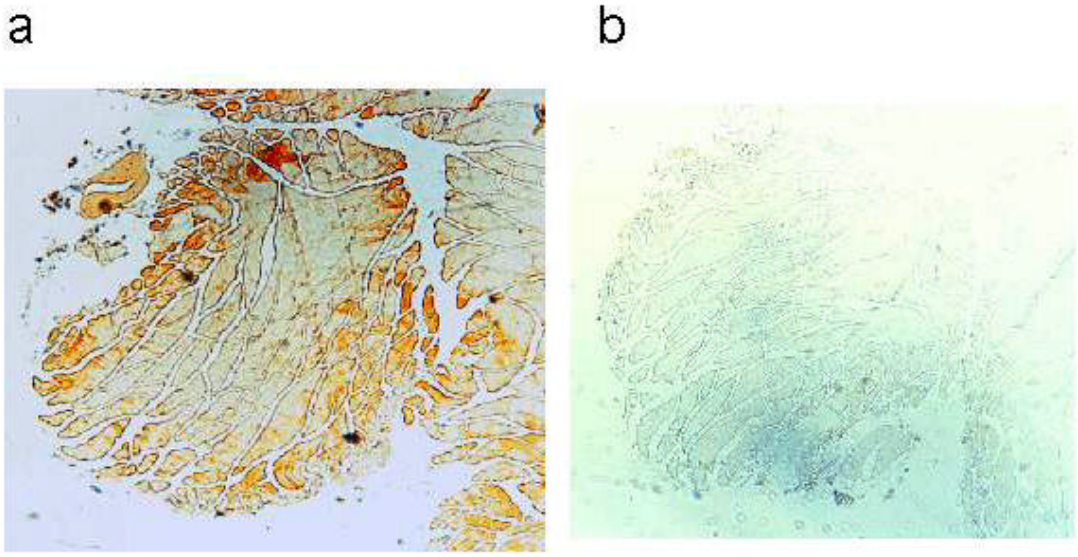



**Figure 7**. Immunostaining against Epo in the muscles injected with serotype 1-derived vectors. The muscle of the animal described in Figure 6 was analyzed. **a**) shows a muscle section stained with 1st and 2nd antibody. **b**) a control without the 1st antibody. Original magnification x 10.

the net expression was comparable to the levels obtained in the muscle transduction. Therefore, the difference may reflect the distribution of transduced cells as the liver is much larger than the tibialis anterior muscle. Although the expression levels of Epo differ significantly among groups, the difference in hemoglobin concentration was relatively modest. The reason seems that as the supraphysiological concentrations were achieved by Epo gene transfer, most animals became unanimously polycythemic. In the cases of lower Epo expression, increase in the hemoglobin levels tend to be slower, due to the presence of the steps to produce red blood cells (typically observed in Figure 2b). On the other hand, in the cases of high Epo expression, increase in the hemoglobin levels were prompt (observed in **Figure 4b**). In this study, we did not measure the functional titers of the vector stocks. It is ideal to quantify the functional titer of each vector stock, and confirm that there is not a discrepancy between these titers. However, there is not a good system to compare the functional titer in a sole system; notably, AAV-4 and –5 have a tropism distinct from the others. Therefore, it is practically impossible to compare functional titers with the same platform. In order to maintain the function of the vector, we paid special attention to the following points. We avoided the use of CsCl as this reagent may affect the functional aspects of the AAV vectors especially AAV-2 (Auricchio et al., 2001). Although there is an increasing number of reports on how to optimize purification strategies for a specific serotype, there is not a purification scheme equally applicable to all of the serotype-based vectors. For this reason, we needed to find a practical method equally applicable to all of the serotypes. In contrast to CsCl, Iodixanol is known to be safe, and can be administered directly to humans. Therefore, we decided to use this reagent in the current study as described (Hermens et al., 1999). Step gradient ultracentrifugation followed by an isopycnic stage resulted in greater purity. In addition, the use of high salt conditions contributes to the purity of the vector stocks, due to a chaotropic action of the chloride ions.

In our study design, we tried not to provoke the immune system in order to compare the relative utility. However, in the case of immunogenic products, there may be a difference in the effect of the immune system. If each serotype-based vector is equally safe, we can choose the optimal type as the vector for a specific target tissue. Our results will guide to design vectors for supplemental gene therapy.

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