INTRODUCTION

Gene therapy is a promising new technology that could be developed to enhance fracture repair and treat bone diseases. Adenoviral and retroviral based gene therapies have proven effective, but safety issues with these therapeutics have not been resolved. Non-viral plasmid-based vectors, on the other hand, provide a much safer form of gene therapy. However, non-viral vectors have not yet been optimized to provide the high levels of transfection in vivo or the long term gene expression provided by viral vectors. There are several barriers to plasmid based gene therapies. These include low levels of gene transfer and lack of tissue specificity. Methods to improve non-viral DNA uptake into the cellular cytoplasm has been improved. However, the subsequent movement of vector DNA into the nucleus has not been adequately addressed. Movement of DNA into the nucleus remains the crucial step for gene transfer and expression in non-dividing and slowly dividing cells. This is an important obstacle to address because bone, like most tissues, consists of slowly replicating or non-replicating cells.

By studying the mechanisms by which viruses efficiently infect cells, it was discovered that some viruses express specific DNA sequences, termed DNA targeting sequences (DTS), that enhance nuclear entry. The first DTS discovered was found in the early promoter of the SV40 virus (1). The SV40 DTS, when present in the viral genome or when added to expression plasmids, is capable of stimulating nuclear entry and significant gene expression in non-dividing cells. When the SV40 genome is injected into the cytoplasm, transcription factors possessing nuclear localization signals bind to the DTS thereby coating the plasmid DNA and directing nuclear entry of the non-viral plasmid-based vector. Through this mechanism efficient transgene expression can be achieved in quiescent cells. Recently a DTS was identified in the SMGA gene promoter and was found to mediate nuclear import of plasmid DNA specifically into smooth muscle cells (2). This indicated that not only can a DTS be used to facilitate more efficient non-viral transgene expression, but it can also be used to create tissue-specific gene therapies.

We hypothesized that identification of a DTS in genes with restricted osteoblast (Ob) expression would allow for creation of a non-viral vector that would possess enhanced nuclear entry and transgene expression and be specifically expressed only in Ob-lineage cells. Currently there is no way to predict if a DNA sequence will act as a DTS. Therefore we tested a series of promoters from genes specifically and robustly expressed in Ob for DTS activity and Ob-specificity. We identified a DTS in the human type I α2 procollagen (hCOL2α2) promoter, which also exhibited robust promoter activity only in Ob-lineage cells. Second, we developed and tested the first prototype skeletal targeting vector with the hRunx2-hCOL2α2 promoter to direct Ob-specific transgene expression in vivo.

METHODS:

Cell Culture. Human, rat, and mouse Obs, chondrocytes, fibroblasts and epithelial cells were maintained in DMEM with 10% CS.

Obbs Promoter Plasmid Vector Construction. Promoter sequences from Ob marker gene promoters were constructed using human genomic DNA from anonymous blood samples, PCR and linked to a luciferase reporter in the pGL3 basic vector. Construction of the hCOL2α2 promoter plasmid was achieved as described (4). The rat Colla1 and mouse OCN promoters were gifts from Dr. Rowe and Dr. Karsenty.

Deletion and Mutation hCOL2α2 Promoter Construct Development. Ob-specific transcription factor binding sites in the hCOL2α2 promoter region (-267 to +45) were identified using bioinformatics. Putative binding site mutations in the hCOL2α2 promoter were made by PCR-site directed mutagenesis. Each mutation construct was tested for promoter activity and nuclear entry activity.

Promoter Activity Assay. Assays were completed as described (4). Microinjection and in situ Hybridization. Plasmid DNA (pDNA) was prepared for each of the Ob-specific promoters. Plasmid DNA was microinjected into the cytoplasm of cells (5). Eight hours after microinjection, the localization of the pDNA was determined by in situ hybridization using a fluorescein-labeled nick-translated probe.

In Vivo Testing of Ob-Specific Non-Viral Vector. To test the specificity of the hRunx2-hCOL2α2 promoter in vivo we used the Cre-loxP technology and created transgenic lines to determine expression patterns in the postnatal skeleton and other tissues. Cre recombinase transgene expression was assessed by crossing hRunx2-hCOL2α2-Cre Tg mice with Rosa26 LacZ reporter mice.

Statistical Analysis. Values are the mean + SEM. Significance was evaluated by the Student’s two tailed t-test. p < 0.05 was significant.

RESULTS:

Each promoter tested demonstrated robust transcriptional activity in Obbs. By contrast, very low levels of activity were detected in chondrocytes, fibroblasts, epithelial cells, and skin cells. When tested for DNA nuclear import activity, only the hCOL2α2 construct showed nuclear import activity (Fig 1). Furthermore, this DTS activity as visualized by nuclear localization was Ob-specific and only occurred in human and rodent Obs. Deletion analysis revealed that the entire 267bp of the proximal promoter were required for nuclear entry and transcriptional activity. Site-directed mutagenesis of putative transcription factor binding sites in the hCOL2α2 promoter indicated that multiple response elements were required for Ob specific nuclear targeting including Nkx and serum response elements. When a portion of the hRunx2 promoter was fused to the hCOL2α2 promoter construct, transcriptional activity was enhanced. Although the hRunx2 promoter has no DTS activity on its own, when fused to the hCOL2α2 promoter nuclear entry is observed. We used this hCOL2α2-based nuclear import sequence together with two copies of an enhancer sequence derived from the hRunx2 promoter to increase Ob specific promoter activity and retain nuclear entry activity. We found that the chimeric hRunx2-hCOL2α2 promoter linked to the luciferase reporter in the pGL3Basic vector demonstrated 2-3 fold more activity than the hCOL2α2 promoter alone in Obbs.

Transgenic mice created with the synthetic promoter demonstrated the in vivo effectiveness of this newly created plasmid construct. We found Cre recombinase activity in distinct cell populations in skeletal tissues, including Obs lining the bone surface and in osteocytes imbedded in mineral. The hRunx2-hCOL2α2-Cre transgenic mouse lines demonstrated the highest levels of Ob specificity and expression compared to hColla2-Cre Tg and mColla1-Cre Tg mice. The hColla2 promoter not only contains a novel Ob-specific DTS that can be used for non-viral expression vector construction to increase transgene expression in skeletal tissues but also acts in vivo to strongly drive specific expression of desired transgenes in Obbs.

DISCUSSION:

We tested several Ob promoters for expression in Obbs and intrinsic nuclear entry activity. We discovered that the proximal hColla2 promoter was most strongly expressed in Obbs and demonstrated Ob specific nuclear entry activity. At least eleven transcription factor binding sites that are conserved in the promoter across species are required for nuclear entry. The chimeric hRunx2-hColla2 promoter can be used to develop nonviral constructs that can be delivered to and expressed only in Obbs. This is the first and crucial step in creating safer non-viral delivery vehicles to provide skeletal-specific gene therapy.

REFERENCES