NEW BIOMEDICAL TECHNOLOGIES

Efficient Expression of the Apolipoprotein A-I Gene Transferred to Human Cells Grown *In Vitro*

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The human apolipoprotein A-I gene controlled by the promoter of the early cytomegalovirus gene was transferred to cultured HeLa cells. The cytoplasmic RNA of transformed cells was shown to contain apo A-I-specific mRNA. Immunoenzyme and immunohistochemical methods revealed the expression of a protein product of the transferred gene in the transformed cells. The amount of synthesized apo A-I protein attained 0.1-0.7% of the total protein of transformed cells.

Key Words: *apolipoprotein A-I gene; biochemical cell transformation; gene therapy of atherosclerosis*

Apolipoprotein A-I (apo A-I) is a major protein component of the high-density lipoproteins of human plasma that participate in the transport of cholesterol. It also acts as a coenzyme of cholesterol-esterifying [7] lecithin-cholesterol-acyltransferase. Apo A-I is responsible for the antiatherogenic properties of the lipoproteins of this class. Moreover, it apparently possesses antiviral [12] and antioxidant [2] properties. In addition, the plasma level of this protein shows an inverse correlation with the acute phase of the inflammatory response of the organism to infection [9]. All these characteristics enable us to consider studies of the transfer of the human apo A-I gene and the attainment of its efficient expression in recipient cells as research aimed at designing methods of gene therapy for the correction of a number of human diseases (mainly atherosclerosis, but possibly also some viral infections). The pilot stage of gene therapy projects that are in progress today involves the transfer of a "therapeutic" gene into cells cultured *in vitro* (biochemical transformation) [10]. If the transferred gene does not integrate into the chromosomes, the situation is referred to as short-term biochemical cell transformation. Short-term biochemical transformation of cells lacking their own apo A-I synthesis by the apo A-I gene has several advantages over stable transformation with the gene, e.g., in cases where periodic compensation of an insufficient apo A-I level near the affected region is required. There are reports concerning short-term as well as stable transformation of mammalian cultured fibroblasts with the human apo A-I gene [3-5,8]. However, in these cases the protein product of gene expression was determined mostly in the culture medium as a result of its secretion by the transformed cells, while the efficiency of gene expression on the cell level was not

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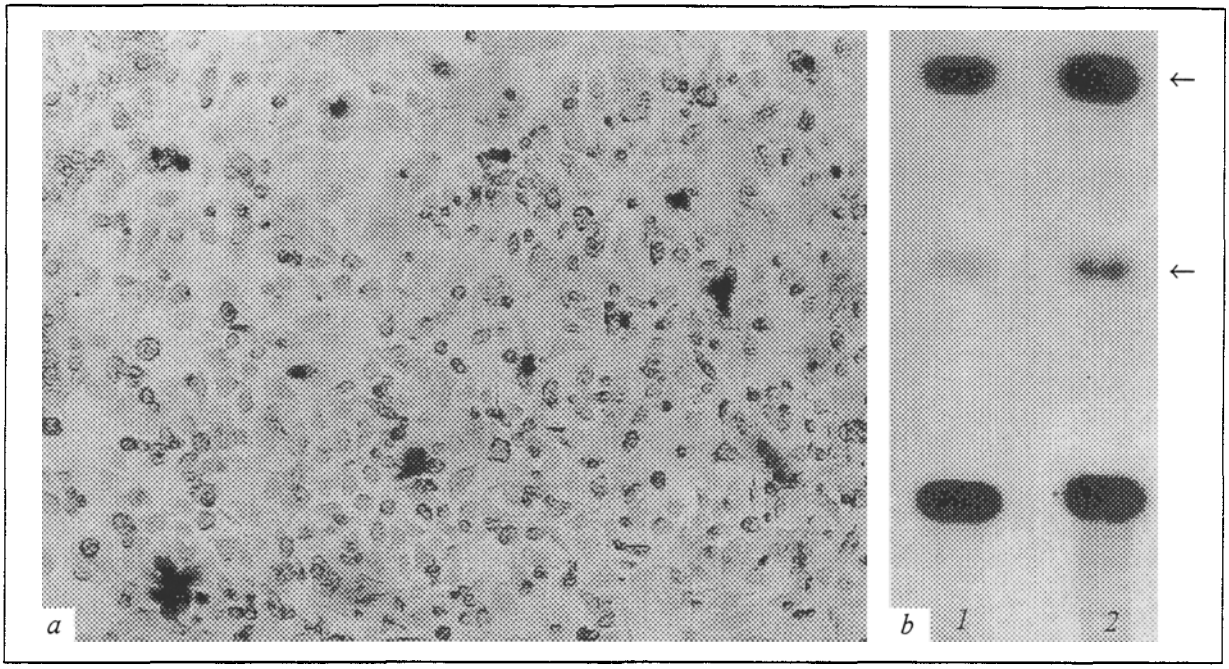

Fig. 1. Evaluation of the transformation efficiency of HeLa cells and activity of cytomegalovirus early gene promoter using bacterial genetic markers. *a*) transformation of HeLa cells with a plasmid containing the bacterial gene LacZ under the control of P_{CMV}. HeLa cells were transformed by the method of calcium–phosphate precipitation and stained via enzymatic reaction using X–Gal as chromogenic substrate. The transformed cells are stained dark. ×125. *b*) estimation of the activity of P_{CMV} linked with the gene of bacterial chloramphenicol acetyltransferase in the Cat test (*2*). As a comparison the vector containing the Cat gene, placed under the control of the promoter of SV40 virus early genes, pSV2–Cat, was analyzed in the same manner (*1*). Arrows point to the positions of chloramphenicol acetylated forms.

studied. In this study we present data pointing to the highly efficient expression of apo A-I in human cells initially lacking this protein.

MATERIALS AND METHODS

Construction of a eukaryotic expression vector based on pUC19 plasmid was performed by conventional methods of genetic engineering.

Cultured HeLa human cells were transformed by the usual procedure of calcium-phosphate precipitation (5 or 10 μg DNA, respectively, for cells grown in 3-cm or 6-cm petri dishes). The efficacy of transformation was estimated by counting the cells stained due to the activity of the transferred bacterial β-galactosidase gene (LacZ gene), using X-Gal as a chromogenic substrate. The activity of the promoter-enhancer regions used was estimated by the Cat test.

Cytoplasmic RNA was isolated by the phenol-detergent method using 10 mM ribonucleoside vanadyl complexes. Isolated RNA was denatured in 1 M glyoxal and analyzed in Northern blotting with a ^{32}P-labeled cDNA probe representing a fragment of the apo A-I gene.

The presence of apo A-I protein in the extracts of transformed cells was detected by means of immunodot-blotting and ELISA; in addition,

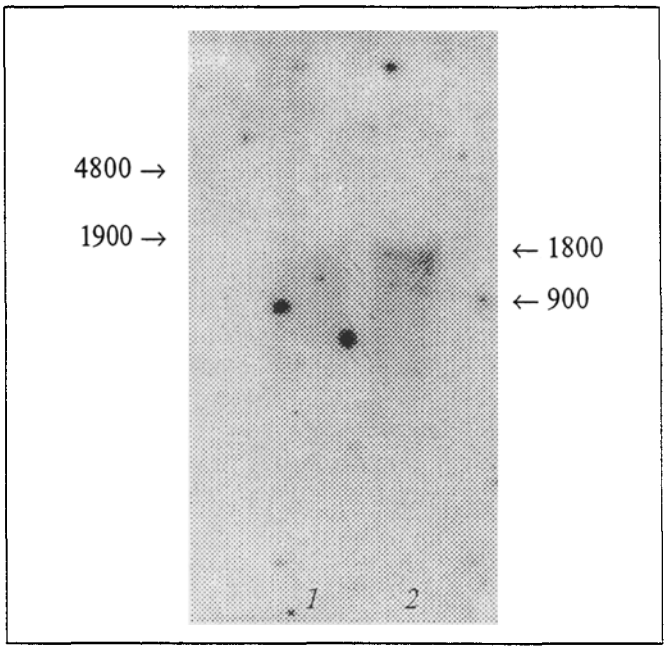

Fig. 2. Identification of human apo A–I–specific RNA by the Northern blot hybridization. Arrows at left indicate the size of the main fractions of mouse liver ribosomal RNA (4800 and 1900 nucleotides) used as markers of molecule size. Arrows at right point to the positions of apo A–I–specific RNA fractions. *1*) cytoplasmic RNA from nontransformed HeLa cells; *2*) cytoplasmic RNA from transformed HeLa cells.

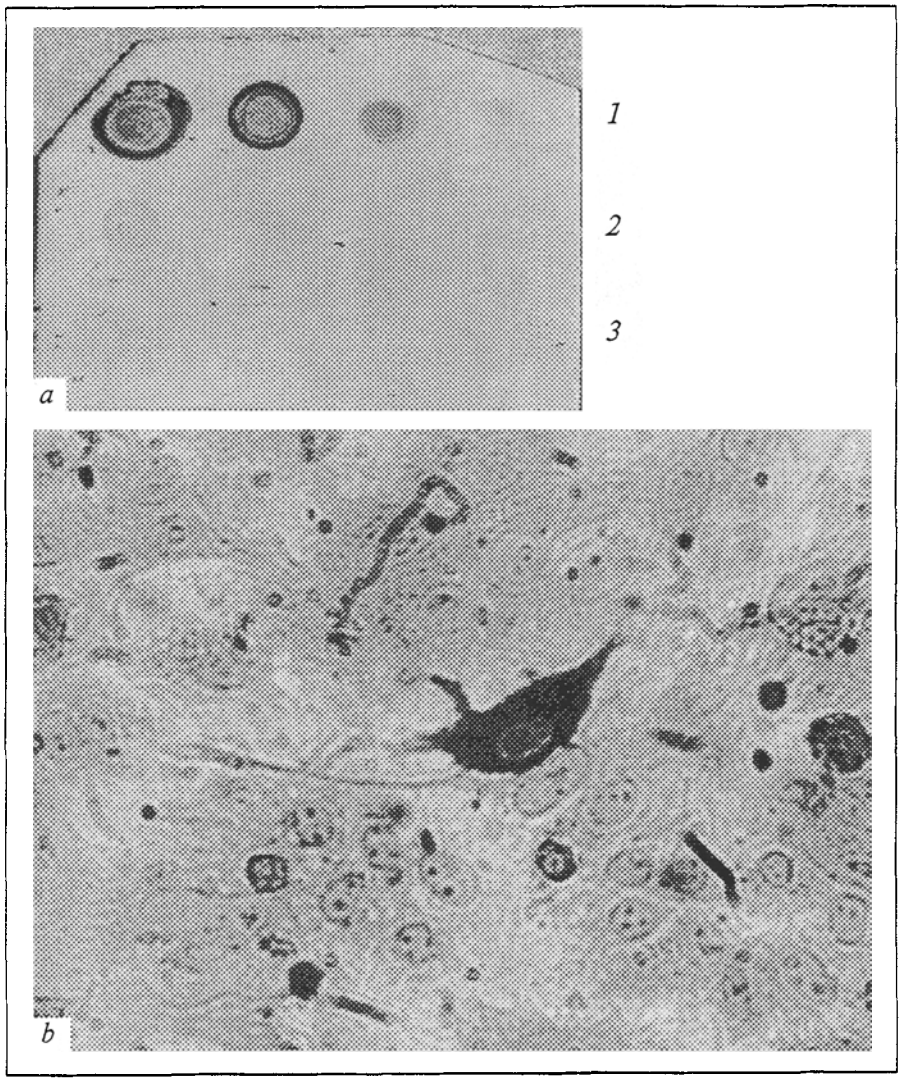

Fig. 3. Immunoenzyme identification of the protein product of the human apo A–I gene in transformed HeLa cells. Polyclonal rabbit antibodies against apo A–I protein were used as first antibodies; the second antibodies represented horseradish peroxidase–conjugated anti–rabbit immunoglobulin; the chromogenic substrate is diaminobenzidine. *a*) analysis of cellular extracts by means of immunodot–blotting; *1*) human serum (positive control); *2*) extracts of transformed cells; *3*) extracts of nontransformed cells (negative control). *b*) immunohistochemical detection of apo A–I protein in transformed cells; protein–producing cells are stained. ×250.

HeLa cells were analyzed immunohistochemically. The following reagents were used in these immunoenzyme assays: rabbit anti-human apo A-I polyclonal antibodies, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies (second antibodies), and diaminobenzidine as a chromogenic substrate.

RESULTS

For the construction of the expression vector we used the 5'-regulatory region (promoter-enhancer) of one of the human cytomegalovirus (CMV) early genes - P_{CMV}. This region efficiently induces the expression of a linked gene in mammalian cells of various types [6]. One of the advantages of this promoter is its constant activity, which is, for example, independent of the proliferative state of a cell, as distinct from other tissue-nonspecific promoters [3]. P_{CMV} was joined to cDNA of the human apo A-I gene containing the polyadenylation signal at the 3'-end. For correct transcription of apo A-I cDNA, the 3'-end of the gene was attached to the region of transcription termination of the human insulin gene, which contains, besides its own polyadenylation signal, other elements necessary for transcription termination and the processing of mRNA [11].

For evaluation of the efficiency of transformation, an analogous vector was constructed in which the human apo A-I gene was replaced by the marker bacterial gene LacZ. The ability of the protein product of the latter gene, μ-galactosidase, to develop staining in the presence of chromogenic substrate was used in our experiments for an estimation of the transformation efficiency (Fig. 1, *a*). The transformation efficiency of HeLa cells proved to be within the range 0.5-1%. For the determination of the CMV promoter efficacy, we constructed an analogous vector with the bacterial chloramphenicol acetyltransferase gene (Cat) substituted for LacZ as a reporter. The results of the

Cat assay (Fig. 1, *b*) showed that the construction obtained expressed the marker gene with high efficiency (as compared to the conventional promoter of SV40 virus early genes), thus confirming that P_{CMV} is one of the most active promoters for mammalian genes [6]. All this provided grounds for using P_{CMV} and the transcription termination region of the human insulin gene to design a vector capable of transferring the apo A-I gene into cultured human cells.

The results of Northern hybridization with cytoplasmic RNA isolated from cells transformed with apo A-I-containing vector are presented in Fig. 2. Two RNA fractions demonstrating intensive hybridization with the radioactive probe were observed, with lengths of 1800 and 900 nucleotides. The fractions are specific to apo A-I mRNA. Due to the vector construction used, these RNAs have similar untranslated 5'-ends and different untranslated 3'-ends, corresponding to the polyadenylation signal of the translation termination region of the insulin gene (1800 nucleotides) and to the polyadenylation signal of the apo A-I gene (900 nucleotides), respectively. These results confirm the need to insert the transcription termination region during the construction of the expression vector [1], including in the case of apo A-I cDNA, in order to achieve more effective gene expression, since in the course of translation both RNA fractions will apparently give rise to the synthesis of identical apo A-I protein molecules.

Immunodot-blotting analysis of cell extracts (Fig. 3, *a*) provided evidence of the synthesis of apo A-I protein by transformed HeLa cells. This result was confirmed by the immunohistochemical analysis of transformed cells (Fig. 3, *b*). Moreover, immunohistochemical data demonstrated the de novo synthesis of apo A-I in transformed cells, since no staining was observed in the control (untransformed) cells under analogous conditions. Quantitative estimation of apo A-I in the transformed cells revealed about 70 ng apo A-I protein per milligram of cellular extract. Taking into account that the frequency of successfully transformed cells varies in the range of 0.5-1%, the concentration of apo A-I in transformed cells will be about 1.4-7.1 μg per mg total cell protein. Thus, the data obtained attest to efficient expression of the HeLa cell-transferred apo A-I gene under the control of the cytomegalovirus promoter. In some experiments the fraction of apo A-I protein attained 0.1-0.7% of the total protein of transformed cells. Keeping in mind that the protein is almost entirely secreted in the growth medium [4,8], we can conclude that the efficiency of our construction, wherein the apo A-I gene functions under the control of P_{CMV}, far surpasses the efficiency of earlier-reported systems [5,8]. The constructed vector of human apo A-I gene expression may be taken as a base for further elaboration of efficient methods of transferring this gene into human cells for the purpose of gene therapy.

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