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Transformation of NIH3T3 Cells with Synthetic c-Ha-*ras* Genes

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Synthetic human c-Ha-*ras* genes in which amino acid codons were altered to those which are frequently used in highly expressed *Escherichia coli* genes were ligated to the 3′-end of Rous sarcoma virus long terminal repeat. When NIH3T3 cells were transfected with the plasmids having those genes with valine at codon 12, leucine at codon 61 or arginine at codon 61, transformants were efficiently produced. These results indicated that the synthetic c-Ha-*ras* genes are expressed in a mammalian system even though their codon usage is altered to correspond with that of *E. coli*. This expression vector system should be useful for studies on the structure-function relationships of c-Ha-*ras*, since the synthetic gene can be easily modified to have multiple base alterations, and can also be used simultaneously for the production of large amounts of p21 in *E. coli* for biochemical and biophysical studies.

Key words: c-Ha-*ras* — Rous sarcoma virus — Long terminal repeat — NIH3T3 cells — Transformation

We have previously reported a total synthesis of the genes for human c-Ha-*ras* proteins (normal c-Ha-*ras* and activated c-Ha-*ras*, having valine at codon 12, leucine at codon 61 or arginine at codon 61) and their expression in *E. coli*.[1,2] Those genes were used for production of large amounts of intact p21 as well as its truncated form for determination of the three-dimensional structure of the catalytic domain of human c-Ha-*ras* p21.[3,4] For future studies on the elucidation of structure-function relationships of the c-Ha-*ras* gene product, p21, the chemically synthesized gene should be very useful, since it can be easily modified to have multiple base alterations by cassette mutagenesis using unique restriction enzyme sites. It is of interest to investigate its biological activity in an *in vivo* mammalian system, since the corresponding c-Ha-*ras* gene product, p21, can be efficiently synthesized in *E. coli* and its biochemical and biological properties can be assayed in parallel. In addition, the synthetic gene can be used for novel *in vitro* mutagenesis experiments in order to investigate the mechanism of activation of the *ras* gene. Therefore, establishment of a mammalian system is necessary and important.

When we designed the synthetic c-Ha-*ras* genes, codons in the c-Ha-*ras* gene were changed to those which are frequently used in highly expressed genes in *E. coli*.[1,2] The question arose as to whether such a change of codon usage would influence expression of the synthetic c-Ha-*ras* genes when they were introduced into mammalian cells. Several *E. coli* genes are known to be expressed in mammalian cells when they are linked to an appropriate mammalian enhancer/promoter sequence.[5–8] Therefore, in order to achieve expression of the synthetic c-Ha-*ras* gene in mammalian cells, a new plasmid containing the synthetic c-Ha-*ras* gene linked with Rous sarcoma virus long terminal repeat (RSV-LTR) was constructed. In this paper, we describe the construction procedure of these kinds of plasmids, and their expression when transfected into NIH3T3 cells.

A plasmid, pRSVneo, in which RSV-LTR is located upstream of the *neo* gene is known to express the *neo* gene efficiently when it is introduced into NIH3T3 cells.[7,8] Thus, pRSVneo was modified by digestion with *Sma* I and *Hind* III, and the large fragment containing RSV-LTR was ligated with *Sal* I linker to yield pRSVΔneo. In order to insert the synthetic c-Ha-*ras* gene, which has *Cla* I and *Sal* I sites at the 5′- and 3′-ends, respectively, a *Cla* I-*Sal* I linker was inserted at the *Sal* I site of pRSVΔneo to produce pRSVΔneo-CS (Fig. 1). After transfection of *E. coli* with pRSVΔneo-CS, the *E. coli* harboring pRSVΔneo-CS was screened by analyzing the restriction map of the plasmid.

Both the plasmid containing the synthetic c-Ha-*ras* gene (pRG12,[2] containing normal c-Ha-*ras* gene) and pRSVΔneo-CS were digested with *Cla* I and *Sal* I. The DNA containing the c-Ha-*ras* gene and the vector derived from pRSVΔneo-CS were purified using agarose gel electrophoresis, and then ligated to form a plasmid carrying the synthetic c-Ha-*ras* gene located downstream

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of RSV-LTR. Figure 1 shows the scheme for construction of pRSV-rg12, which contains the normal c-Ha-*ras* gene. A similar procedure was carried out to obtain expression vectors containing activated c-Ha-*ras* genes [pRSV-rv12 (valine at codon 12), pRSV-r161 (leucine at codon 61), pRSV-rr61 (arginine at codon 61)].

The DNA transfection assay was performed by the calcium phosphate procedure as reported previously.[9, 10] The plasmid DNA containing the c-Ha-*ras* gene was co-precipitated with 30 μg of carrier NIH3T3 DNA, added to NIH3T3 cells (8×10^5 cells), and incubated at 37°C for 5 h. Then, 17% glycerol was added in order to increase the efficiency of DNA incorporation. Transfected cells were cultured in 10 ml of Dulbecco's modified Eagle's medium with 5% bovine calf serum. After incubation for 2 to 3 weeks, the cells were stained with Giemsa's solution (Merck), and the formed foci were counted.

It has been reported by many investigators that point mutation of c-Ha-*ras* as well as c-Ki-*ras* and N-*ras*, which cause amino acid substitutions at codon 12 or 61 of the gene product, p21, are responsible for transforming ability when they are introduced into NIH3T3 cells.[11-18] It is now understood that such point mutations of the *ras* family genes are actually involved in initiation and/or progression of many human cancers.[17, 18]

In a preliminary experiment, the transforming activity of pRSV-rv12 containing the synthetic c-Ha-*ras* (Val-12) gene was compared with that of pT24, which contains a 6.2 kb fragment of human activated c-Ha-*ras* 1 gene (Val-12) derived from T24 bladder carcinoma cell lines.[19] As shown in Table I, pRSV-rv12 was approximately twice as efficient in focus-forming activity compared with pT24, per ng of DNA. Since the chain length of pT24 is twice that of pRSV-rv12, this result indicates that the transforming activity of pRSV-rv12 is similar to that of pT24 on a molar basis, showing that the synthetic c-Ha-*ras* gene, which was designed for production of p21 in *E. coli*, is expressed sufficiently in rodent cells under the control of RSV-LTR. Next, plasmids containing normal and other activated c-Ha-*ras* genes [pRSV-rg12 (Gly-12, Gln-61), pRSV-r161 (Leu-61) and pRSV-rr61

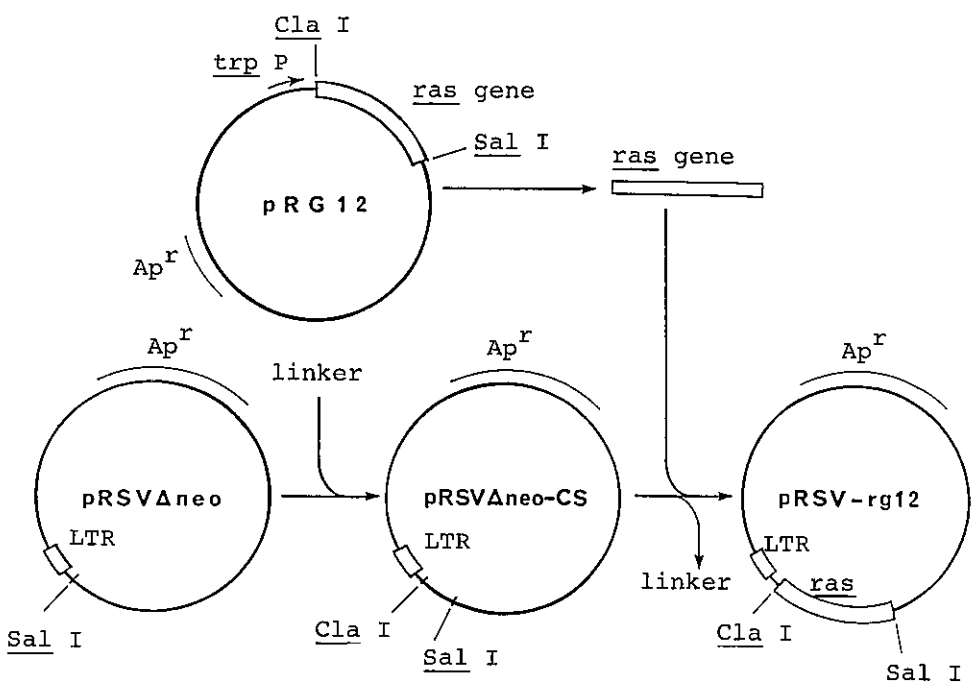

Fig. 1. Construction of a plasmid carrying a synthetic c-Ha-*ras* gene located downstream of RSV LTR. Vector plasmid pRSVΔneo was digested with *Sal* I. A *Cla* I-*Sal* I linker (the sequences of *Cla* I-*Sal* I linker upper strand and lower strand are 5′ dTCGAATCGATGGGGAAAAG 3′ and 5′ dTCGACTTTTCCCCATCGAT 3′, respectively) was inserted into the vector. This plasmid, termed pRSVΔneo-CS, was digested with *Cla* I and *Sal* I and plasmid containing the synthetic c-Ha-*ras* gene for normal p21 and *E. coli* tryptophan promoter was also digested with *Cla* I and *Sal* I, following by isolation of the *ras* gene. The vector and the c-Ha-*ras* gene were ligated to form a new plasmid pRSV-rg12, which contains the normal c-Ha-*ras* gene downstream of RSV-LTR. A series of LTR-*ras* plasmids (pRSV-rv12, pRSV-r161 and pRSV-rr61) were constructed by the same procedure.

Table I. Transforming Activity of Synthetic c-Ha-*ras* Genes

Plasmid	Amino acid at position in the Ha-*ras*-coded p21		Transforming efficiency (foci/ng DNA)
	12	61	
pRSV-rv12 (synthetic)	Val	Gln	36.1[a]
pT24 (genomic)	Val	Gln	16.8[a]
pRSV-rg12	Gly	Gln	0[b]
pRSV-rv12	Val	Gln	37.4[b]
pRSV-r161	Gly	Leu	22.0[b]
pRSV-rr61	Gly	Arg	28.2[b]

a) 15 ng of plasmid DNA was used.
b) 5 ng of plasmid DNA was used.

(Arg-61)] were transfected. Table I shows the result when 5 ng of plasmid DNA per plate was used for the transformation assay. All activated c-Ha-*ras* genes were found to have similar transforming activity. On the other hand, pRSV-rg12 which contains the synthetic normal c-Ha-*ras* gene did not induce any focus when transfected into NIH3T3 cells. Therefore, it was concluded that this LTR-synthetic *ras* gene system is useful for expression of the synthetic *ras* gene and a good assay system for detection of transforming ability of mutated genes.

It was previously reported that normal human c-Ha-*ras* gene with a strong promoter is able to transform NIH3T3 cells, indicating that production of large amounts of normal p21 in the cells is itself sufficient for the induction of transformation.[20] Failure to obtain transformed foci with the synthetic normal c-Ha-*ras* gene under the present experimental conditions is perhaps due to inefficient translation of the synthetic gene, because of its codon usage which is unfavorable in a mammalian system. However, it is clear that p21 is produced sufficiently, because the activated synthetic *ras* genes have focus-forming activity. Therefore, it is suggested that the synthetic c-Ha-*ras* genes are transcribed efficiently under the control of RSV-LTR while translation of the genes is slow because of their unfavorable codon usage.

The synthetic gene can be easily modified to alter several amino acid residues in coded p21 at once, since parts of the nucleotide sequence of the gene can be easily exchanged.[1,2] In the case of site-directed mutagenesis of the natural c-Ha-*ras* gene (either genomic DNA or cDNA), it is rather difficult to perform such drastic alteration of the gene. We are now carrying out such manipulations in order to investigate the functional role of amino acid residues in important domains of the c-Ha-*ras* protein p21. The three-dimensional structure of truncated p21 has been recently elucidated.[3,4] Based on this information, it should be possible to alter several important amino acid residues in the GDP/GTP binding domain for investigation of the structure-function relationships of p21. Because the synthetic c-Ha-*ras* gene has several unique restriction enzyme sites, it can be used for novel *in vitro* mutagenesis experiments and transfected directly into NIH3T3 cells. Therefore, in order to study the mechanism of activation of the *ras* gene, the system as reported here should be very useful. Experiments dealing with these subjects are in progress.

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