Genes and enzymes of the acetyl cycle of arginine biosynthesis in \textit{Corynebacterium glutamicum}: enzyme evolution in the early steps of the arginine pathway

Vehary Sakanyan,\textsuperscript{1} Pavel Petrosyan,\textsuperscript{2} Michèle Lecocq,\textsuperscript{1} Anne Boyen,\textsuperscript{3,4} Christiane Legrain,\textsuperscript{5} Marc Demarez,\textsuperscript{5} Jean-Noël Hallet\textsuperscript{1} and Nicolas Glansdorff\textsuperscript{3,4,5}

Author for correspondence: Vehary Sakanyan. Tel: +33 40373065. Fax: +33 40292042.

A cluster of arginine biosynthetic genes of \textit{Corynebacterium glutamicum} ATCC 13032, comprising \textit{argI}, \textit{argB} and \textit{argD} as well as part of \textit{argC} and \textit{argF}, has been cloned by heterologous complementation of an \textit{Escherichia coli argE} mutant. The gene order has been established as \textit{argCJBD} by sequencing the entire 4.4 kb cloned DNA fragment. The \textit{C. glutamicum argB} gene can be transcribed in \textit{E. coli} cells from an internal promoter located in the coding part of the preceding \textit{argI} gene, whereas transcription of the \textit{argI} gene appears vector-dependent. Expression of the corynebacterial \textit{argB} gene is repressed by arginine in the native host but not in recombinant \textit{E. coli} cells. Feedback inhibition of the corresponding \textit{N-acetylglutamate} kinase activity was observed both in cell extracts of \textit{C. glutamicum} and in recombinant \textit{E. coli argB} auxotrophic strains. Extracts of \textit{E. coli} cells carrying cloned corynebacterial DNA display an ornithine acetyltransferase activity (encoded by \textit{argJ}) which alleviates the acetylornithinase (encoded by \textit{argE}) deficiency of the enterobacterial host. In contrast to \textit{Bacillus} \textit{stearothermophilus} ornithine acetyltransferase which also exhibits acetylglutamate synthase activity, \textit{C. glutamicum} ornithine acetyltransferase appears monofunctional. \textit{ArgA} and \textit{ArgB} proteins from different sources share highly significant similarities. The evolutionary implications of these data are discussed.

Keywords: \textit{Corynebacterium glutamicum, argCJBD} sequence, ornithine acetyltransferase, \textit{N-acetylglutamate} kinase, regulation

\textbf{INTRODUCTION}

Arginine biosynthesis sets off with the acetylation of the amino group of glutamate (Fig.1), mediated by \textit{N-acetylglutamate} synthase (acetyl\textit{CoA}:l-glutamate \textit{N-acetyltransferase}; EC 2.3.1.1; the \textit{argA} gene product). This acetylation prevents spontaneous cyclization and hence proline formation during the subsequent modifications of the 5-carboxyl group. Two strategies have evolved for the terminal removal of the acetyl group. In the so-called linear pathway operative in \textit{Enterobacteriaceae} and in the archaeon \textit{Sulfolobus solfataricus} acetylornithinase (\textit{N\textsuperscript{2}-acetyl-l-ornithine} amidohydrolase; EC 3.5.1.16; the \textit{argE} gene product) catalyses the hydrolysis of \textit{N-acetylornithine} into the arginine precursor ornithine and acetate (Cunin \textit{et al.}, 1986; Van de Casteele \textit{et al.}, 1990). All other prokaryotes investigated up to now, including methanogenic archaea (Meile \& Leisinger, 1984; Van de Casteele \textit{et al.}, 1990), \textit{Neisseria} \textit{gonorrhoeae} (Shinners \& Catlin, 1978), members of the genus \textit{Bacillus} (Sakanyan \textit{et al.}, 1992) and the eukaryotic microbes (Davis, 1986) use the more economical cyclic pathway that was first brought to light in \textit{Micrococcus} \textit{glutamicus} (Udaka \& Kinoshita, 1958), in which the acetyl group is recycled with generation of acetylglutamate. The transacetylase between acetylornithine and glutamate is mediated by the \textit{argF} gene product, ornithine acetyltransferase (\textit{N\textsuperscript{2}-acetyl-l-ornithine}:l-glutamate \textit{N-acetyltransferase}; EC 2.3.1.35). In organisms depending on the cyclic pathway, \textit{N-acetylglutamate} synthase therefore fulfills an anaplerotic function.

The EMBL accession number for the sequence reported in this paper is X86157.
Complementation experiments with N. gonorrhoeae (Picard & Dillon, 1989; Martin & Mulks, 1992) and Bacillus stearothermophilus (Sakanyan et al., 1990) revealed that one small DNA fragment of these organisms could complement both argA and argE auxotrophs in E. coli. The responsible genes have since been sequenced and sufficient genetic (Martin & Mulks, 1992; Sakanyan et al., 1992) and enzymic (Sakanyan et al., 1993a) data have been accumulated to prove that these organisms possess a bifunctional argJ-encoded product capable of using both the acetyl group of N-acetylornithine and that of acetylCoA to acetylate glutamate. It appears that Bacillus subtilis also harbours a bifunctional acetyltransferase (O'Reilly & Devine, 1994).

Although the argJ gene by itself would thus be able to assure both the first and the fifth steps of arginine biosynthesis in these organisms, there is genetic evidence for the existence of an independent functional argA gene in N. gonorrhoeae (Picard & Dillon, 1989; Martin & Mulks, 1992) and enzyme data for B. stearothermophilus point in the same direction (Sakanyan et al., 1992). In Pseudomonas aeruginosa, however, the synthase and transacylase activities can be separated by gel filtration (Haas et al., 1972). Moreover, argA mutants have been isolated which display a normal acetyltransferase but no synthase activity and hence an arginineless phenotype. It has been shown that the synthase enzymes of P. aeruginosa (Haas et al., 1972; Haas & Leisinger, 1974) and Saccharomyces cerevisiae (Wipf & Leisinger, 1979) lack ornithine acetyltransferase activity; the properties of the ornithine acetyltransferases were not studied in detail. The data for P. aeruginosa therefore suggest the existence of a monofunctional ornithine acetyltransferase in this organism. Similarly, the cloned Streptomyces coelicolor ornithine acetyltransferase gene complements E. coli argE but not argA mutants (Hindle et al., 1994).

In some micro-organisms the metabolic flow through the acetyl cycle is controlled by arginine-mediated feedback inhibition of the second biosynthetic step, catalysed by N-acetylglutamate kinase (ATP: N-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8; Udaka, 1966; Hoare & Hoare, 1966; Haas & Leisinger, 1975; Meile & Leisinger, 1976).
### Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain/plasmid</th>
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<td>[F' traD36 proAB lacIΔZ∆M15]</td>
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**Plasmids**

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### METHODS

#### Bacterial strains and plasmids.

The strains and plasmids used in this work are listed in Table 1.

#### Media and growth conditions.

*C. glutamicum* cells were grown on a rotary shaker (150 r.p.m.) at 30 °C inuria-Bertani (LB) medium (Sambrook et al., 1989) or in a synthetic medium described by Bröer et al. (1993). LB as well as the synthetic M9 medium (Miller, 1972) were used for *E. coli* K12 strains. M9 was supplemented with all auxotrophic requirements other than arginine for complementation analysis of arg mutants by *C. glutamicum* DNA. *E. coli* cells were grown at 37 °C, except for complementation tests which were performed at 30 °C. Antibiotics were added at a final concentration of 50 μg ml⁻¹ for ampicillin, 30 μg ml⁻¹ for chloramphenicol and 25 μg ml⁻¹ for tetracycline.

#### DNA manipulation and transformation.

Chromosomal DNA of *C. glutamicum* was isolated as described by Eikmanns et al. (1991). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method of Birnboim & Doly (1979) or with Qiagen-tip columns (QIAGEN). Agarose gel electrophoresis, DNA restriction, alkaline phosphatase treatment and ligation were performed following classical protocols (Sambrook et al., 1989). *E. coli* strains were transformed following the CaCl₂ procedure (Sambrook et al., 1989) or by electroporation with the geneZapper 450-2500 apparatus (International Biotechnologies) according to the manufacturer's recommendations.

#### DNA sequencing and sequence analysis.

Prior to sequencing subfragments were cloned into the pBluescript II KS(+) vector.
ExoIII-generated unidirectional deletions were obtained according to the method of Sambrook et al. (1989). Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using sequencing Quick-denaturate plasmid sequencing kits (USB) and [α-32P]dATP (Amersham). Either universal or reverse primers or synthetic 17-mer oligonucleotides (provided by D. Gigot, Research Institute, CERIA) were used. Sequence nucleotides were synthesized and the sequence data were compiled, analysed and aligned with various programs from the MacVector package (International Biotechnologies), as well as with CLUSTAL (Higgins & Sharp, 1988) and FASTA (Pearson & Lipman, 1988). The Swiss-Prot database (EMBL) was consulted for protein sequences.

**Enzyme assays.** *E. coli* and *C. glutamicum* cells were grown in synthetic media, harvested by centrifugation during the exponential phase, washed in 0.9% (w/v) NaCl and resuspended in 10 mM potassium phosphate buffer (pH 6.5) containing 15% (v/v) glycerol, 1 mM EDTA, 1 mM DTT and 2 mM PMSF for N-acetylglutamate synthase and ornithine acetyltransferase assays, or in 25 mM Tris/HCl buffer (pH 7.5) for other assays. Cells were disrupted by sonication and the resulting crude extract was centrifuged (20000 g, 15 min). All these treatments were performed at temperatures below 10 °C. Enzyme assays were carried out at 30 °C. For inhibition experiments the crude cell extracts were passed through Sephadex G-25 columns equilibrated with extraction buffer.

N-Acetylglutamate synthase and ornithine acetyltransferase were assayed as described in Van de Casteele et al. (1990), except that 15% (v/v) glycerol, 10 mM MgCl₂, and 7 mM amino-oxic acid were added to the incubation mixture. N-Acetylglutamate kinase was measured by the ferric chloride method (Udaka, 1966) as described previously by Van de Casteele et al. (1990). Acetylornithinase was measured by the method of Vogel & McLellan (1970) as described in Sakanyan et al. (1993c).

**RESULTS AND DISCUSSION**

**Cloning of *C. glutamicum* arginine biosynthetic genes and their expression in *E. coli* mutants**

Cloning of the *C. glutamicum argJ* gene was undertaken by selecting for heterologous complementation of argE deficiency in *E. coli* as obtained with *N. gonorrhoeae* and *B. stearothermophilus* DNA (Picard & Dilon, 1989; Sakanyan et al., 1990). An EcoRI-digest of *C. glutamicum* ATCC 13032 DNA was ligated into EcoRI-cleaved vector pBR327. The resulting plasmids were transformed into *E. coli* K12 XSIΔ2R and arginine prototrophs were selected on synthetic medium supplemented with succinate, ampicillin and tetracycline. The recombinant pPP2 plasmid which carries a single 4.4 kb EcoRI insert was isolated from the selected transformants. Its restriction map derived from single- and double digest data with several enzymes is shown in Fig. 2.

Ampicillin-resistant pPP2 transformants of various arginine auxotrophic *E. coli* K12 mutants were screened for complementation by replica plating on synthetic medium without arginine. Apart from the argE-deficient strain mentioned above, pPP2 complemented argB mutant XB25. Of particular interest to our study is its failure to complement argA-deficient strains XA4 and its derivative XA4argE. Enzyme assays summarized in Table 2 confirmed these results. N-Acetylglutamate kinase and ornithine acetyltransferase activities measured in cell extracts of *E. coli* K12 XB25(pPP2) and *E. coli* XA4(pPP2), respectively, were raised significantly above the background level of the plasmidless strain. Acetylglutamate synthase and acetylornithinase activities, in contrast, remained undetectable in extracts of *E. coli* K12 XA4(pPP2) and *E. coli* K12 XSIΔ2R(pPP2). From these results it can be inferred that the cloned fragment harbours the structural *C. glutamicum* argB and argJ genes and that the argJ-encoded ornithine acetyltransferase relieves the acetylornithinase deficiency of the *E. coli* argE mutant by heterologous complementation.

Since the native *C. glutamicum* ATCC 13032 displays N-acetylglutamate synthase activity (see Table 2) it can be assumed that the corresponding argA gene is not located on the pPP2 plasmid unless it is non-functional in *E. coli* cells. Consequently, the cloned *C. glutamicum* argJ gene appears to encode a monofunctional enzyme capable of transacylating the acetyl group of *N*-acyetylornithine (ornithine acetyltransferase activity), but not that of acetylCoA (N-acetylglutamate synthase activity) to glutamate.

**Sequence analysis**

The sequence of the entire 4.4 kb insert of *C. glutamicum* DNA in pPP2 has been established on both strands using ExoIII-generated 200–300 bp deletions in a subcloned 1.9 kb HindIII–XhoI fragment (plasmid pKS-1.9) and several other subcloned fragments. Analysis of the nucleotide sequence reveals five large ORFs (Fig. 3) oriented in the same way, those at the fragments’ ends being truncated. The ORFs were numbered as described previously by Van de Casteele et al. (1990) and Dilon (1989). Two of the five ORFs, ORF2 and ORF3, correspond to arginine biosynthetic genes truncated ORF1 matches the C-terminal end of the argC-encoded polypeptide, ORF2, ORF3 and ORF4 appear respectively homologous with the argJ, B- and D-encoded enzymes and finally, the truncated ORF5 corresponds with the N-terminal region of the argF gene product. Putative RBSs can be found upstream of the proposed initiation codons of ORFs 2–5 and are indicated in Fig. 3. The cloned 4.4 kb insert therefore seems to contain an important part of a large arginine biosynthetic cluster as in *B. subtilis* (Moutain et al., 1986), *N. gonorrhoeae* (Picard & Dilon, 1989), *B. stearothermophilus* (Sakanyan et al., 1990; Sakanyan et al., 1993a) and *S. coelicolor* (Hindle et al., 1994). A fairly large ORF6 oriented in the opposite direction showed no significant resemblance to any sequence as yet registered in the protein databases. Searching specifically for homology between *C. glutamicum* ORFs and the known acetylglutamate synthase sequences of *E. coli* (Brown et al., 1987), *P. aeruginosa* and *Pseudomonas putida* (Dharmshtiti & Krishnapillai, 1993) and *Neurospora crassa* (Y. Yu & R. L. Weiss, unpublished; EMBL accession number L35484) was negative, except for ORF3 which...
corresponds to the argB gene. This homology between acetylglutamate synthase and acetylglutamate kinase is supported by the comparison of other argA and argB gene couples (Reith & Munholland, 1993; see below).

According to a BESTFIT comparison, the C. glutamicum ArgJ sequence shares 36, 35 and 39% identical amino acids with the ornithine acetyltransferases of N. gonorrhoae (Martin & Mulks, 1992), B. subtilis (O'Reilly & Devine, 1994) and B. stearothermophilus (Sakanyan et al., 1993a), respectively. The CLUSTAL alignment shown in Fig. 4 indicates that the similarity covers the whole sequence, though the C. glutamicum polypeptide appears shorter than its bifunctional homologues by 11–12 amino acids at the N-terminal end. Its predicted molecular mass is 39.8 kDa, approximately 3 kDa less than for the other known bacterial ornithine acetyltransferases; it contains only 67% of basic amino acids which is substantially lower than the 10% found for the other ArgJ products.

A BESTFIT comparison shows that the N-acetylglutamate kinase polypeptide sequence of C. glutamicum shares approximately 42% identical amino acids with the N-acetylglutamate kinase of Porphyrmycymbus (Reith & Munholland, 1993), 39% with that of B. stearothermophilus (Sakanyan et al., 1993b), 35% with that of B. subtilis (O’Reilly & Devine 1994), 29% with that of E. coli (Parsot et al., 1988), 25% with that of N. crassa (Gessert et al., 1994) and 23% with those of S. cerevisiae (Boonchird et al., 1991) and Schizosaccharomyces pombe (Van Huffel et al., 1992).

Sequence analysis indicates that the initiation codon of the C. glutamicum argD gene is contiguous to the argB TAA termination codon, whereas an intergenic space is observed between the other corynebacterial arg genes of the cluster. The same tendency was observed in B. stearothermophilus (Sakanyan et al., 1993b) and B. subtilis (O’Reilly & Devine, 1994), where the argB and argD genes overlap for a few nucleotides and suggests translational coupling, while a relatively long intergenic space was found at the argC/argJ and argJ/argB transitions. A CLUSTAL alignment of the deduced ArgD polypeptide sequence with those of E. coli, B. subtilis and S. cerevisiae (data not shown) revealed 40, 42 and 36% identical amino acids, respectively.

The overall G+C content of the 4.4 kb stretch of C. glutamicum DNA amounts to 54.6%, which corresponds with the mean value for glutamic-acid-producing corynebacteria (Yamada & Komagata, 1970). A remarkable feature in codon usage is the unexpectedly high appearance of the rare CGA arginine codon in the argJ gene (4 out of 12 codons). Apart from that there is a relatively low occurrence of G+C in the third position of the argJ codons: 51.7% as compared to 59.3 and 64.1% for the argB and argD genes, respectively. There is no strong bias for corynebacterial ‘preferred’ codons (Malumbres et al., 1993) in either argJ, argB or argD; therefore a low-to-moderate expression might be expected.

**Evidence for a secondary promoter upstream of the argB gene**

From the sequence analysis it appears that the pEX4 plasmid, constructed by subeloning the 3.1 kb EcoRI-Xhol fragment in EcoRI/SalI-cut pUC9 vector, should carry the whole of the structural information for both the
Fig. 3. For legend see facing page.
Fig. 4. Comparison of entire amino acid sequences of ornithine acetyltransferases by CLUSTAL v alignment. C. glutamicum (this work); B. subtilis (Sakanyan et al., 1993a); B. subtilis (O'Reilly & Devine, 1994); N. gonorheroeae (Martin & Mulks, 1992). Asterisks indicate that identical residues occur in all four polypeptides; dots show replacement by similar amino acids.

Transcription of the C. glutamicum argB gene in E. coli, however, seems independent from an extraneous promoter, a result confirmed by the significant N-acetylglutamate kinase activity observed in the E. coli K12 XB25(pGAB4) strain (Table 2). The pGAB4 plasmid was constructed by inserting the 1.9 kb HindIII-XhoI fragment (see Fig. 2) in HindIII/SalI double-digested promoter–probe vector pGA46. As no transcription can proceed from the pGA46 vector into the inserted DNA (An & Frisen, 1979), it seems likely that in E. coli cells at least, transcription can be initiated at a promoter located between the HindIII site at 1172 nt and the beginning of the structural argB gene of C. glutamicum. Evidence for the occurrence of a promoter site recognized by E. coli RNA polymerase preceding the argB structural gene has been reported for B. stearothermophilus as well (Sakanyan et al., 1993b).

Repression of enzyme formation and feedback inhibition by arginine

N-Acetylglutamate synthase, N-acetylglutamate kinase and ornithine acetyltransferase activities were measured in extracts of C. glutamicum cells grown in the absence or in the presence of arginine (Table 2). The levels of N-acetylglutamate synthase and ornithine acetyltransferase were not affected by arginine addition. A fivefold repression of N-acetylglutamate kinase synthesis was observed in the presence of arginine.

Inhibition of arginine activity by arginine was tested for the three enzymes by adding L-arginine (concentration range 0.01–100 mM) to the reaction mixture of the enzyme assays described above. Arginine was found to inhibit N-acetylglutamate synthase and N-acetylglutamate kinase activities; the arginine concentrations for 50% inhibition were 40 and 2 mM, respectively. Feedback inhibition by arginine of corynebacterial N-acetylglutamate kinase has already been reported (Udaka, 1966). Inhibition by L-arginine (more than 80% at 10 mM arginine) could also be detected for C. glutamicum N-acetylglutamate kinase synthesized in E. coli K12 argB mutant cells carrying pPP2, pKS-1-9 or pGAB4. Whether N-acetylglutamate kinase inhibition actually plays a regulatory role in vivo remains to be established, since the apparent inhibition constant of arginine is high. One must, however, take into account that this value was determined with crude extracts and at saturating levels of substrates.

Inhibition by ornithine was tested for the enzymes listed in Table 2, except acetylornithinase. No inhibition could be detected for the first or the second biosynthetic enzymes, either in C. glutamicum or in recombinant E. coli cell extracts. The corynebacterial ornithine acetyltransferase activity was inhibited by ornithine (product inhibition); the apparent Ki value at saturating levels of substrates was 5 mM.

Fig. 3. Nucleotide sequence of the 44 kb DNA region of C. glutamicum ATCC 13032 with the predicted amino acid sequences encoded by the argCIBDF genes cluster. The potential ribosome-binding site (RBS) and selected restriction sites referred to in Fig. 2 are underlined. Stop codons are marked with asterisks.
Table 2. Specific activities of four arginine biosynthetic enzymes in C. glutamicum and in E. coli strains carrying C. glutamicum arg genes

Values are the means of at least two measurements made on independent cultures. The values for replicate assays differed from the mean by < 20% for N-acetylglutamate synthase and ornithine acetyltransferase, and by < 10% for the other activities.

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ND, Not determined.

* Arginine was added to synthetic medium at a concentration of 5 mM. Succinate (0.5%, w/v) was added for E. coli XS1D2R strains. E. coli strains carrying plasmids were grown in the presence of ampicillin.

Evolutionary relationships between enzymes of the acetyl cycle

Comparison based on the Pearson & Lipman algorithm (1988) fails to reveal any significant similarity between the sequences of C. glutamicum ornithine acetyltransferase and known N-acetylglutamate synthases. This result enforces our earlier suspicion that in spite of their functional relatedness the argA and argJ gene products belong to different evolutionary families (Sakanyan et al., 1993a).

Fasta comparisons (Pearson & Lipman, 1988) with the registered polypeptide sequences do not point to any clear affiliation of the ornithine acetyltransferases. On the other hand similarities between the N-terminal part of ArgA and the ArgB polypeptide sequence have recently been noticed (Reith & Munholland, 1993; Gessert et al., 1994). These similarities appear in fact most pronounced when E. coli ArgA and C. glutamicum ArgB amino acid sequences are compared: application of the Pearson & Lipman (1988) RDF2 program establishes a similarity value that is 15 standard deviations above the mean value obtained with 100 random permutations of either sequence, a highly significant value. It may therefore be assumed that acetylglutamate synthases and kinases are indeed evolutionarily related.

An alignment of the known ArgA and ArgB polypeptide sequences (data not shown) indeed reveals several highly conserved amino acids. Surprisingly, no direct relatedness could be detected between the acetylglutamate synthase of N. crassa (Y. Yu & R. L. Weiss; EMBL accession number L35484) and those of other organisms: the N. crassa ArgA enzyme seems weakly similar to kinase sequences only.

The other known acetylglutamate synthase polypeptide sequences are obviously longer than the kinases by a stretch of about 100–140 amino acid residues, and some highly conserved regions occur in this part. The results of a Fasta search of the registered sequences in the SwissProt database suggests an intriguing affiliation. Indeed, a high similarity between this region of the E. coli acetylglutamate synthase and a 153 amino acid ORF, found next to the trpGDC cluster in Azospirillum (Zimmer et al., 1991), which in its turn can be related to the E. coli Rim1 enzyme that acetylates the N-terminus of the ribosomal S18 gene (Yoshikawa et al., 1987) and the Streptomyces lavendulae StaT protein, an acetylCoA-dependent acetyltransferase (Horinouchi et al., 1987). The Azospirillum ORF is, moreover, highly similar to the E. coli PhnO protein (Makino et al., 1991), which is involved in alkylphosphonate utilization that again appears related to various other acetyltransferases. These similitudes might reflect an ancient link between the argA-encoded enzyme C-terminal part and a family of small acetylCoA-dependent acetyltransferases.

New insights into the function and the evolution of the acetyl cycle are presently being looked for in a comparative analysis of structure–function relationships.
in the monofunctional ornithine acetyltransferases of \textit{C. glutamicum} disclosed in this work and the bifunctional, homologous enzyme of \textit{B. stearothermophilus} (Sakanyan et al., 1993a).

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V. SAKANYAN and OTHERS


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