CHEMICAL SYNTHESIS AND ENZYMATIC ASSEMBLY OF FRAGMENTS OF THE DNA CODING IMMUNODOMINANT EPITOPES OF HUMAN IMMUNODEFICIENCY VIRUS*

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More than twenty 13- to 55-membered oligo(poly)nucleotides have been synthesized by the H-phosphonate solid-phase method in the manual variant using an original procedure. From the oligonucleotides obtained, seven DNA duplexes coding immunodominant epitopes of HIV-1 proteins have been formed: 598-609 and 737-748 gp41, 91-115 and 105-115 gag, and 940-951 pol and a number of its mutants. The ligase assembly and polymerization of the DNA duplexes obtained has been carried out. The efficiency of ligation amounted to from 60 to 90%. The possibility of their directed polymerization was determined by the flanking of the duplexes by partially completed half-sites of restrictases BamHI and XhoI.

The genetic-engineering construction of proteins with a given immunospecificity permits not only the production of highly immunogenic drugs having practical value but also the investigation at the molecular level of the dependence of the immune response on the primary structures of individual sections of a protein [1-3]. One of the methodological approaches to such construction consists in the introduction of synthetic oligonucleotides coding selected immunodominant epitopes of the proteins under investigation into a definite part of a carrier-protein gene [4]. In individual cases, the directed immune response caused by a hybrid protein can be intensified by the multiplication of an epitope introduced into it [2, 5] which is achieved, in particular, by the polymerization of the duplexes coding the oligonucleotides.

In the present paper we described the chemical-enzymatic synthesis of monomeric and oligomeric variants of oligonucleotide duplexes coding sequential antigenic determinants of HIV-1 proteins: LGIWGCSGKLIC (598–609 gp41) [6, 7], GERDRDRSIRL (737–747 gp41) [8, 9], EEEQNKSKKKA (105–115 gag) and EALDKIEEEQNKSKKKA (99–115 gag) [10, 11] and VYYRDSRNPL (941–950 pol) [12] and a number of its mutants (Fig. 1). The primary structures of the oligonucleotides synthesized are shown in Fig. 1.

In planning the syntheses, as a basis we selected codons used preferentially by E. coli [13]. For introduction into plasmids containing the gene of the carrier protein, the duplexes (see Fig. 1) were flanked by incomplete half-sites of restrictases BamHI and XhoI. The use of such sticky ends in ligation prevents the appearance of inverted repeats, determining the possibility of the directed polymerization of the duplexes. For the performance of controlled polymerization it was proposed to use terminating oligonucleotides. Since it is known that T4 DNA ligase can "link" oligo(poly)nucleotides while "ignoring" mononucleotide deletions and noncomplementary units in the composition of the substrate [14], in order to terminate polymerization it was necessary to modify both 5'-ends of the duplex undergoing ligation. In the variant used here, the oligonucleotide terminating the polymerization of the (+)-chain was deprived of the 5'-terminal nucleotide unit while, conversely, the terminator of the (−)-chain contained at its 5'-end an additional noncomple-

*Abbreviations adopted: HIV-1 – human immunodeficiency virus, type 1; (+)-chain – the coding chain – and (−)-chain the chain complementary to the coding chain of the DNA duplex; CPG – controlled-pore glass; PAAG – polyacrylamide gel; Py – pyridine.

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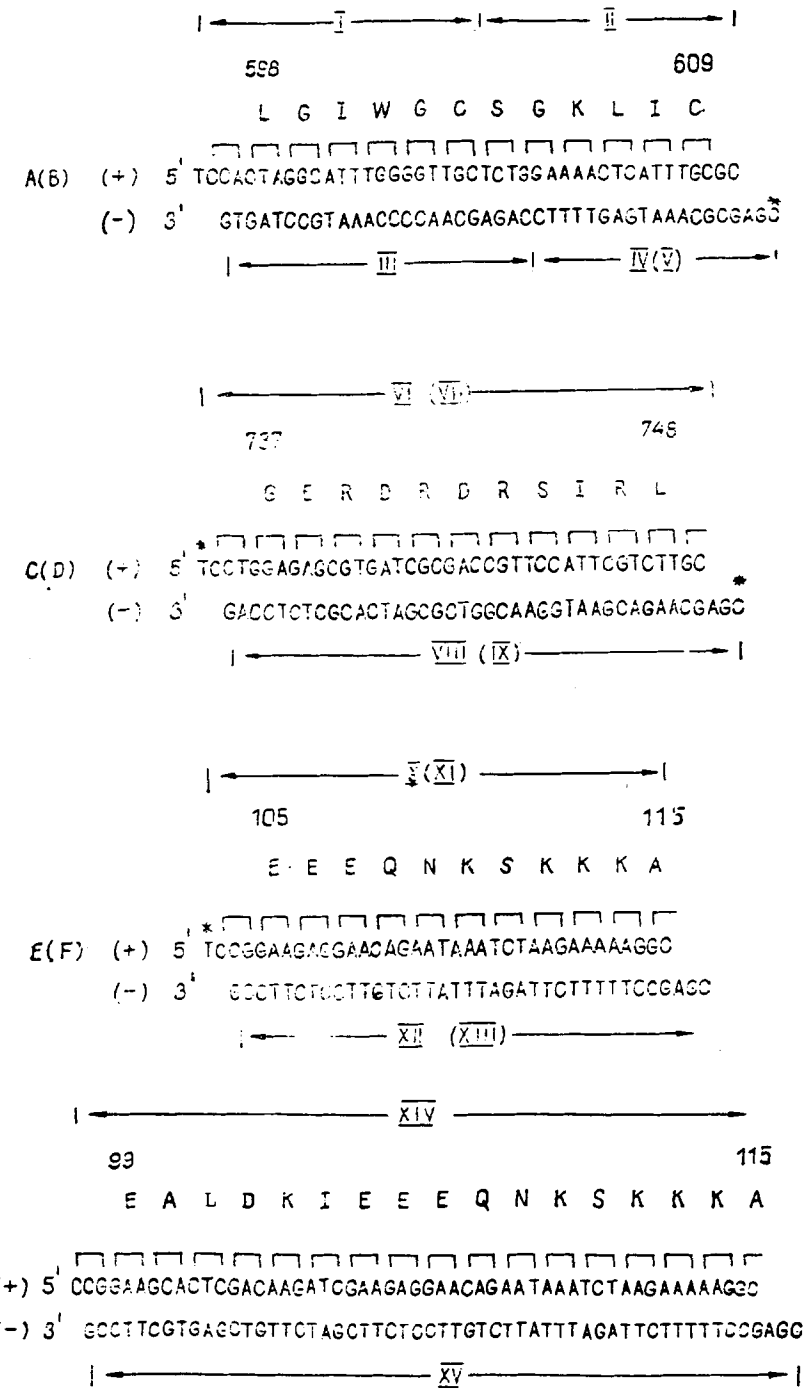

Fig. 1 (continued on p. 330).

mentary nucleotide, as proposed in [15]; thus, the polymerization of both chains was excluded. Before the inclusion of the monomeric or polymerized synthetic duplexes flanked by polymerization terminators into the plasmid vector linearized by treatment with BamHI and XhoI, the partial completion of its sticky ends was required.

Oligonucleotides (I)-(XXIV) (see Fig. 1) were synthesized by the solid-phase H-phosphonate method [16] in the manual variant in plastic microcolumns, the scales of synthesis amounting to from 0.2 to 1.3 μmole and the yield at the condensation stage being 97-98%. After deblocking, the oligonucleotides were isolated by preparative electrophoresis in 8-20% PAAGs containing 7 M urea. The primary structures of the oligonucleotides were confirmed by Maxam-Gilbert sequencing [17] using the modifying conditions described in [18].

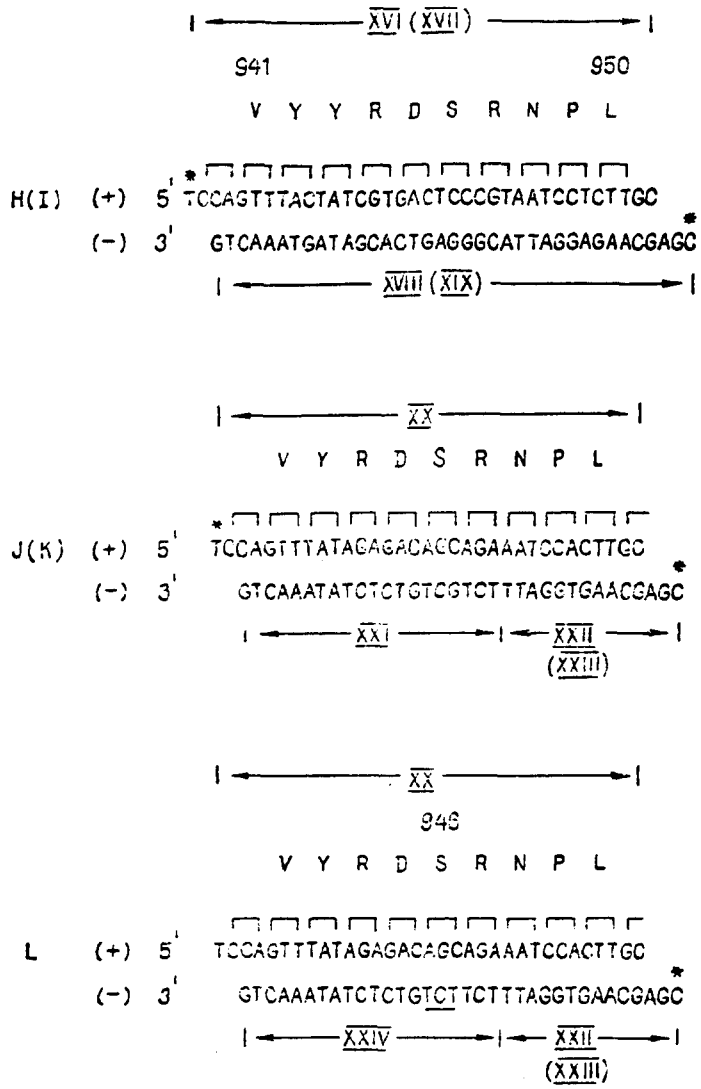

Fig. 1. Primary structures of the DNA duplexes coding native and modified sequences from HIV-1 proteins: gp41 (duplexes A-D), gag (E, F, and G), and pol (H and I; and, with the deletion of Tyr^{943} – J and K). Duplex L contains a noncomplementary pair in the Ser^{946} codon which, on cloning, must lead to the appearance of clones with the native sequence from the pol protein and clones in which Ser^{946} has been replaced by Arg. The designations of the polymerization-terminating oligonucleotides (see text) and of the duplexes formed with their participation are given in parentheses. The nucleotide members absent from the oligonucleotides terminating the polymerization of the (+)-chains and additionally introduced into the terminators of the polymerization of the (–)-chains are marked by asterisks.

Ligase assembly and polymerization were investigated separately for the (+) and (–)-chains of the duplexes with the use as substrate of nonkinated (and, consequently, nonligating) oligonucleotides of the complementary chain. For the quantitative determination of the efficacy of ligation, kination was carried out with [γ-^{32}P]ATP, with the completion of kination by ATP, and the yield of ligation products was calculated as the percentage of the total radioactivity of all the ^{32}P-containing bands in PAAG. Results on the efficacy of ligation are given in Table 1. As can be seen from the table, the use in the assembly of the chains of oligonucleotides containing modified 5'-ends (see, for example, the assembly of the (–)-chains of duplexes B and K) completely excludes the possibility of polymerization.

TABLE 1. Assembly and Polymerization by T4 Ligase of DNA Duplexes Coding Immunodominant Epitopes of HIV-1 (see Fig. 1)

DNA duplex*	Chain ligated	Efficacy of the assembly of the chain, %	Efficacy of the polymerization of the chain of the duplex, %	
			dimer	oligomers
A	(+)	9	7	45
A	(−)	14	12	70
B	(−)	91	0	0
C	(+)	—	40	25
C	(−)	—	60	15
J	(+)	—	8	86
J	(−)	35	0	55
K	(−)	84	0	0

*The total nucleotide concentration was from 10^{-4} to 10^{-3} M; the oligonucleotides were taken in equimolar amounts in a 1.5- to 2-fold excess with respect to the 5'-^{32}P-labeled component.

Thus, a chemical-enzymatic synthesis of DNA duplexes coding native and modified immunodominant epitopes of HIV-I proteins that it is proposed to incorporate into plasmid vectors with the aim of obtaining in E. coli hybrid proteins with a given immunospecificity has been effected.

EXPERIMENTAL

The pyridine, methylene chloride, acetonitrile, and 1-methylimidazole were Merck products, absolutized; the acrylamide, N,N'-methylenebisacrylamide, and triazole were Sigma products; the pivaloyl chloride and the monomethoxy- and dimethoxyl tritile chloride were Fluka products; and the other reagents were of domestic production. The nucleoside H-phosphonates were obtained by the method of [16].

Synthesis of Oligonucleotides by the H-Phosphonate Method. The nucleoside H-phosphonates were dried by two evaporations with absolute CH_3CN and then in vacuum (0.1-0.2 mm Hg) over P_2O_5 for 12-15 h. Monomers were dissolved in a mixture of absolutized Py/CH_3CN (1:1), and the 0.2-0.25 M solutions were added dropwise to plastic test-tubes separately in each stage of the growth of the oligonucleotide chain. CPG-500 with a long aminoalkyl "stalk" (Electro-Nucleonics) was used as the support, and the grafting of the first nucleotide unit was carried out by the method of [20], the load amounting to 15-25 μmole/g.

Synthesis was performed in plastic tips to 1-ml automatic pipettes with siliconized glass-wool filters. The solvents were added to the column by means of 2-ml glass syringes, the deblocking mixture (2.5% solution of trichloroacetic acid in CH_2Cl_2) by means of a plastic syringe, and the solutions of the nucleoside H-phosphonate and pivaloyl chloride (1.25 M in Py—CH_3CN (1:1)) by means of a 200-μl automatic pipette. The reagents and solvents were forced through the column by a current of air from the compressed-air supply that had been dried by passage through a calcium chloride tube. At the condensation stage, the outlet from the column was closed, and the polymer was stirred hydraulically after the column had been attached to an automatic pipette. A cycle of chain growth lasted for 180-230 s, including 50-60 s for condensation and 50-70 s for detritylation. Before and after condensation, the column was washed with absolute CH_3CN, the total volume of washing being 3-3.5 ml per stage.

The Deblocking and Isolation of the Oligonucleotides. An oligonucleotide was oxidized on a polymer as described in [16]. The polymer was washed with Py and $CHCl_3$ and dried with air, after which the tip of the column was sealed, the polymer was covered with 200 μl of concentrated aqueous ammonia, and ammonolysis was carried out in a stainless-steel test autoclave at 65°C for 12-15 h. After ammonolysis, the tip of the column was cut off, the solution of oligonucleotide that had filtered through the glass wool was lyophilized, and the dried residue was dissolved in 1 M KOAc and reprecipitated with ethanol as described in [21].

5'-Terminal ^{32}P labels were introduced into the oligonucleotides by T4 polynucleotide kinase, as in [22]; ligation was carried out at 6°C in a buffer containing 66 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM ATP for 12 h. After ligation, the reaction mixtures were analyzed by electrophoresis in 12-20% PAAGs containing 7 M urea. The yield of ligation products in each experiment was determined from the radioactivity of the corresponding zone as a percentage of the total radioactivity of all the ^{32}P-containing zones of the given track.

The primary structures of the oligonucleotides were confirmed by the Maxam-Gilbert method [17]. Modification was carried out in solution: for G – with dimethyl sulfate at pH 3.5, 20°C, 10 min; for A + G – with formic acid, 20°C, 20 min; for T + C – with hydrazine, 20°C, 25 min; for C – with hydrazine and NaCl, 20°C, 20 min, as in [18]. Sequencing electrophoresis was performed in 22% PAAG containing 7 M urea.

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