TRANSFORMATION OF SUBSTRATE SPECIFICITY OF EcoR1* RESTRICTASE BY GLYCEROL

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In the presence of glycerol, EcoR1 restriction endonuclease hydrolyzes DNA into a larger number of fragments than under ordinary conditions. On the addition of glycerol to 50% concentration, this enzyme begins to act by what is called the "EcoR1' type" of restriction, which was producible experimentally only by reducing the ionic strength and increasing the pH of the solution. However, under these extremal conditions, the enzyme is quickly inactivated and does not give reproducible results, especially during the hydrolysis of high-molecular-weight DNA. The conditions found for manifestation of "EcoR1' activity" yield reproducible results, which is essentially equivalent to the discovery of a new "restriction endonuclease."

KEY WORDS: *restriction endonuclease; hydrolysis of DNA of phages and adenoviruses; restriction site.*

Restriction endonucleases of type II (restrictases) have been widely used to study the structure of genomes [1] because of their high specificity and their ability to hydrolyze palindromic nucleotide sequences of DNA. EcoR1 restrictase was isolated from strain RU 13, carrying the factor of multiple drug resistance RTF1 [8]. Under conditions of high ionic strength (100 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$) and at pH 7.3-7.4 the enzyme "recognizes" the hexanucleotide sequence IAATTC, and forms breaks four nucleotide pairs apart in each of the DNA strands. At the site of hydrolysis, so-called sticky ends appear in the DNA fragments under these circumstances [3]. With a decrease in the ionic strength to 25 mM Tris-HCl, 2 mM $MgCl_2$, and with an increase in pH to 8.5 the specificity of the enzyme is changed ("R1' activity"), as a result of reduction of the restriction site from 6 to 4 nucleotides [4].

However, under these extremal conditions the enzyme is quickly destabilized, so that reproducible results cannot be obtained, especially in the analysis of DNA of high molecular weight.

The object of this investigation was to seek stable and reproducible conditions for the modification of EcoR1 activity.

EXPERIMENTAL METHOD

EcoR1 restrictase was isolated from strain RU 13 by a modified scheme [7]. The enzymic reaction was carried out in medium containing 0.07 M Tris-HCl buffer (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, and 7 mM 2-mercaptoethanol for EcoR1 restrictase and in 0.025 M Tris-HCl, 2 mM $MgCl_2$, and 7 mM 2-mercaptoethanol (pH 8.5) for the detection of EcoR1' activity. DNA was isolated from plasmid Col E1, bacteriophages λ, Sd, and T_7, human adenoviruses of types 1 and 6, and monkey adenovirus type 7 by the phenolic deproteinization method of Landy et al. [2].

*The nomenclature of the restriction enzymes follows that of Smith and Nathans [6].

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TABLE 1. Effect of Glycerol on Degree of Fragmentation of DNA by EcoR1 Restrictase

Source of DNA	Mol. wt. of DNA, millions of daltons	Number of DNA fragments on hydrolysis with EcoR1				Upper and lower limits of molecular weight of DNA fragment formed, millions of daltons		
		a	b	c	d	a	b	d
Phage λ	30	6	23	26	29	13,7 2,1	3,0 0,3	3,0 0,3
Phage Sd	55	5	26	24	28	28 0,3	20 0,2 1,6	7 0,2 1,6
Phage T_7	30	Not hydrolyzed	11	12	12	30	0,2	0,2
Human adenovirus: type 1	23	3	24	18	26	17 1,6	6,0 0,3	6,0 0,3
type 6	23	4	29	29	30	17,6 1,05	7,0 0,3	7,0 0,3
Monkey adenovirus: type 7	23	2	36	36	40	12 10	12 0,5	4,3 0,5
Plasmid Col E1	4,3	1	10	10	10	4,3	4,3 0,1	4,3 0,1

Legend: a) under ordinary conditions: 70 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, and 7 mM 2-mercaptoethanol; b) under EcoR1' conditions [4]; c) under ordinary conditions with addition of 50% glycerol; d) under EcoR1' conditions with addition of 50% glycerol.

EXPERIMENTAL RESULTS

The addition of glycerol to the reaction mixture modified the activity of EcoR1 restriction endonuclease. With an increase in the glycerol concentration in the medium to over 20% the number of DNA fragments formed rose sharply, but the most stable results of hydrolysis were obtained only with 50% glycerol. To judge from the results given in Table 1, the pattern of hydrolysis of DNA in 50% glycerol and under "EcoR1' conditions" [4] is virtually identical (fractionation in 0.8% agarose) both in molecular-weight distribution of the restriction fragments formed and in their number. However, analysis in polyacrylamide–agarose copolymer may reveal additional fragments (details are not given). The degree of DNA hydrolysis (complete or partial) was judged visually from the disappearance of high-molecular-weight partial hydrolysis products. The small differences in the number of fragments and in their size under the conditions now used and under those recommended by Polisky et al. [4] during electrophoresis in agar gel are attributable mainly to the partial hydrolysis observed under nonoptimal conditions of pH and ionic strength. This view stems from the idea that, both under the present conditions and those described by Polisky et al. [4], all the "EcoR1' sites" are equally sensitive to hydrolysis by the modified enzyme. On the one hand, this possibility is confirmed by the fact that the degree of fragmentation of Col E1 DNA in the presence of 50% glycerol or at alkaline pH values in medium with low ionic strength, and also during their combined action, is identical. On the other hand, in the case of the remaining DNA, not all "EcoR1' sites" are equally accessible for the action of the modified enzyme, and during the additive action of pH and ionic strength and of 50% glycerol the number of DNA fragments formed is greater than under "EcoR1' conditions" and with the glycerol system. High-molecular-weight partial hydrolysis products are degraded under these conditions to fragments of lower molecular weight. On the basis of these facts it can be postulated that 50% glycerol, especially together with "EcoR1' conditions," may influence the accessibility of particular sites for hydrolysis by restrictase. The addition of heterologous DNA to the "EcoR1' system" [4] 30 min after the beginning of incubation did not lead to hydrolysis of that DNA, evidence of inactivation of the enzyme at an alkaline pH and in a low ionic strength.

Meanwhile, in a system with 50% glycerol, heterologous DNA is fragmented even if added 4 h after the beginning of the reaction.

Under ordinary conditions of EcoR1 hydrolysis with small quantities of the enzyme the picture of so-called partial hydrolysis of DNA appears, when besides complete hydrolysis products, intermediate products of the reaction are also present. In concentrations below 20%,

glycerol promotes partial hydrolysis, i.e., it inhibits the enzymic reaction. Glycerol is evidently an allosteric effector for EcoR1 restrictase and affects the conformation of the enzyme and its substrate specificity. The effect discovered means that fragmentation of DNA through the action of EcoR1 restrictase can be deliberately controlled. DNA fragments arising under the influence of EcoR1 in the presence of 50% glycerol have "sticky ends," for the writers have shown that their covalent "cross-linkage" by DNA polynucleotide ligase is possible. This fact opens up new prospects for the construction of genomes and production of hybrid DNA molecules. Hopeful results have already been obtained for the effect of various organic solvents on EcoR1 activity.

The data given are the mean results of five experiments. The relative deviation was ±5%. The volume of the incubation medium was 20 μliters and the DNA content 1 μg. The quantity of enzyme added was between 1 and 3 conventional units. The reaction time was 1 h and the temperature 37°C. The reaction was stopped by the addition of EDTA to the medium up to a final concentration of 0.05 M. DNA restriction fragments were analyzed by electrophoresis in agar gel and polyacrylamide–agarose copolymer [5]. The gels were stained in a solution of ethidium bromide (1 μg/ml) and photographed in ultraviolet light. To calculate the molecular weight of the DNA fragments formed, an internal standard was used, namely EcoR1 fragments of DNA from bacteriophage λ. The values for the molecular weight of the restriction fragments of DNA from phage λ were taken from Thomas and Davis [7].

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