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Cleaving DNA at Any Predetermined Site with Adapter-Primers and Class-IIS Restriction Enzymes

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A four-component system has been designed that makes it possible to prepare a double-stranded (ds) DNA fragment; one fragment end is predesigned (by the use of a class-IIS restriction enzyme and adapter-primer), and the other end corresponds to any normal restriction cut. The system is composed of the phage M13mp7 single-stranded (ss) target DNA; the Fok I restriction enzyme; an oligodeoxynucleotide adapter-primer, which permits one to introduce Fok I cuts at any specified site in the target DNA; and DNA polymerase, which converts the ss target into a ds form ready for cloning. In this system, the oligodeoxynucleotide adapter-primer serves several purposes. The 5′ hairpin ds domain of the adapter-primer contains the Fok I recognition site. Its 3′ ss domain selects a complementary site on the target ss DNA, hybridizes with it to form the ds cleavage site, and serves as a primer to convert the ss M13mp7 target to ds DNA.

THERE IS A LIMITED NUMBER OF restriction enzymes, with which it is possible to cleave DNA only at preexisting recognition sites (*1*). It would be of great advantage, however, to be able to cut DNA at any predetermined site. To attain this goal it is necessary to use a combination of two moieties, one with the DNA site specificity and another with the DNA cleaving function. We have used a specially designed oligodeoxynucleotide adapter and a class-IIS restriction enzyme, which permitted a precise cut between any predetermined two nucleotides of the single-stranded (ss) DNA target (*2–4*). Other investigators have used either an oligodeoxynucleotide (*5, 6*) or site-specific DNA binding protein (*7*) to guide the cutting complex to the specific site on DNA; the target DNA was cut either chemically, by EDTA·Fe complex (*5, 7*), or by an otherwise nonspecific nuclease (*6*). In these three methods (*5, 7*), the DNA cuts were imprecise and statistically distributed around the target sequence. Our present system produces double-stranded (ds) DNA fragments with one predetermined and precisely cut end.

Most of the class-II restriction endonucleases (*1*) recognize a specific sequence on ds DNA and cleave it at specific points within this sequence. Subclass IIS (*2*) cuts DNA, not within the recognition sequence, but at a precise distance from it on the same molecule. This physical separation between the recognition and cleavage sites permitted us to design a system (*2, 3*) where the Fok I recognition site and one of the cuts are on an adapter oligodeoxynucleotide, whereas the second cut site is on M13mp7 ss target DNA.

In the present study we have tried to redesign our system (*3*) so as to obtain defined ds DNA fragments, ready for cloning, instead of just introducing cuts in the ss target DNA at the predetermined sites. The general design of the new four-component system, as adapted for the Fok I enzyme, is shown in Fig. 1. The 34-mer adapter has a 5′-$\overset{GGATG}{CCTAC}$ Fok I recognition site within its ds hairpin domain and a 14-nucleotide (nt) ss domain; the latter is complementary to nt 1339–1353 of the M13mp7 (*4*) ss target DNA. This ss domain plays two roles in our system: (i) when paired with M13mp7 ss DNA, it provides a ds cut site for Fok I (indicated by vertical arrows in Fig. 1A) and (ii) it serves as a primer for PolIk-catalyzed DNA synthesis starting at its 3′-OH terminus, using M13mp7 ss target DNA as a template, and thus creating the ds M13mp7 molecule with a novel predesigned restriction site for the Fok I enzyme (Fig. 1A). The products of this novel Fok I cut are shown in Fig. 1B. The cohesive ends are filled in by PolIk, as shown by the italicized letters in Fig. 1B.

The sizes of the expected Fok I digestion products are summarized in Fig. 2. The replicative form (RF) ds DNA of M13mp7 is cut into 218-, 910-, 2775-, and 3335-bp fragments by Fok I (see the outer ring of Fig. 2 and lanes 6 and 7 of Fig. 3). The 34-mer adapter would induce a new cut between nt 1341 and 1342 of the M13mp7 (+) strand, and thus a new 1116-bp fragment should be produced (Fig. 2) shortly after the intitiation of the adapter-primed, PolIk–catalyzed DNA synthesis. Indeed, as shown in Fig. 3, a new 1116-bp fragment appeared as a major digestion product within 10 minutes after the start of DNA synthesis (lane 2); this fragment was totally absent in lanes 6 and 7, which represent the Fok I–cleaved RF ds DNA of M13mp7. As DNA synthesis proceeded, the regular products (analogous to those in lanes 6 and 7) of Fok I cleavage appeared, first the shortest (218-bp) fragment and later the 910-bp, 2775-bp, and finally the 3335-bp products (lanes 2 through 5), in the same order as they are primed by the 34-mer adapter-primer oligodeoxynucleotide (Fig. 2). Actually, the restriction patterns in Fig. 3 are somewhat more complex, because the 34-mer has a secondary binding site on M13mp7 DNA, including the second cut next to nt 6938 (Fig. 2) (*3*). This cut would generate a 603-bp fragment (Fig. 2), which is present in the form of an early appearing 603-bp minor

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band in Fig. 3, lanes 2 through 5. Also the novel 307-bp and 2219-bp fragments, which are postulated in Fig. 2, do show up as very minor late-appearing bands in over-exposed autoradiograms (Fig. 3).

The precision of the Fok I adapter-primer–induced cut was assessed by the sequencing of the 5′ end of the 1116-bp fragment (Fig. 4). We conclude that the expected 5′-GGTAGCAA . . . end constitutes either the only adapter-primer–induced product or the very major product, since no bands appear in the sequencing gel that could correspond to any other cut next to the 1341/1342 cut. This result, in conjunction with the appearance of very sharp bands of a 1116-bp fragment in Fig. 3 (lanes 2 through 5), indicates that the Fok I adapter-primer–induced cut must be very precise.

Only one of the four PolIk enzyme preparations tested (all from various commercial sources) was satisfactory in our experiments, because the three others converted the ss M13mp7 template to ds RF DNA, even when no adapter-primer was added. No ds DNA bands appeared when the adapter-primer was absent and our selected New England Biolabs PolIk preparation (*8*) was used together with all other reaction components (Fig. 3, lane 1). The other PolIk preparations were probably contaminated

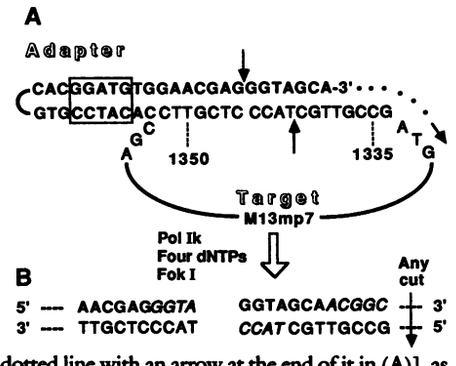

Fig. 1. The design of the four-component system [adapter-primer, ss target DNA, Fok I enzyme, and PolIk (Klenow fragment of *Escherchia coli* DNA polymerase I) + four deoxyribonucleoside triphosphates (dNTPs)] for obtaining ds DNA fragments with one predetermined end. (**A**) The adapter is a 34-mer oligodeoxynucleotide with a 10-bp hairpin ds domain carrying the Fok I recognition site (boxed) and a 14-nt ss domain complementary to nt 1339–1352 (*3, 4*) of the phage M13mp7 ss target DNA. In our earlier experiments (*3*), this 34-mer adapter was directing the Fok I–mediated cleavages, as shown by vertical arrows. (**B**) In the present experiments, the 3′-end of the 34-mer adapter-primer was elongated by the PolIk enzyme and all four dNTPs [dotted line with an arrow at the end of it in (A)], as detailed in the legend to Fig. 3, thus converting the M13mp7 ss target to the ds RF DNA. Addition of Fok I resulted in staggered cleavages [see also vertical arrows in (A)], creating a predetermined end, the position of which depended solely on the design and sequence of the adapter-primer. Fok I also produced other cuts (with the nearest one represented by the downward arrow near the right end) in the ds M13mp7 molecule (Fig. 2), generating a ds fragment composed of two 1116-nt strands with 4-nt cohesive ends. Because of the presence of PolIk and four dNTPs, the cohesive ends were filled in (see italicized letters), resulting in a 1120-bp blunt-ended fragment. However, those filled-in ends that carried the adjoining Fok I recognition site (not shown here) were again cut by this restriction enzyme, releasing a tetranucleotide (*12*). When Fok I was inactivated at 70°C and PolIk was re-added, it was possible to obtain only the filled-in blunt-ended products.

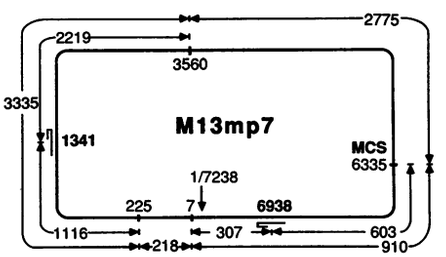

Fig. 2. Restriction map of the RF ds DNA of phage M13mp7 (*4*). The heavy line represents the phage genome with Fok I sites at nt 7, 225, 3560, and 6335. The sizes (in base pairs) of the resulting four fragments are shown on the outermost ring. The primary (nt 1341) and secondary (nt 6938) cuts induced by the 34-mer adapter (represented by a partial hairpin symbol; see also Fig. 1A) create new fragments of 1116 nt (1120 bp after fill-in) and 603 nt, as shown. The 2219- and 307-nt fragments are produced when PolIk reaches the ends of the ss template, previously cut by Fok I at the nt 1341 and 6938 sites. MCS designates the multiple cloning site, and the 1/7238 arrow shows the conventional beginning and end of the M13mp7 genome (*4*).

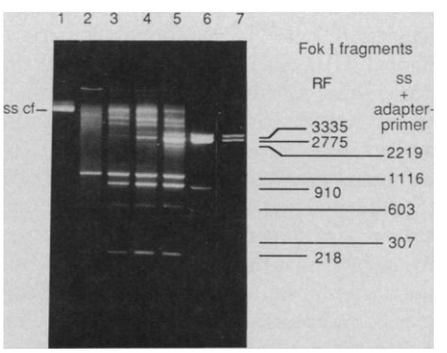

Fig. 3. Synthesis of specific DNA fragments with a predetermined terminus. The outline of the experiment is shown in Fig. 1 and the expected sizes of fragments in Fig. 2. Lanes 1, 6, and 7 are controls and represent the closed ss circular form (ss cf) of M13mp7 DNA (lane 1), and four ds products (218, 910, 2775, and 3335 bp) of Fok I digestion of the RF M13mp7 ds DNA (lane 6, 2 μg of DNA; lane 7, 1 μg of DNA) (*8*). Lanes 2 through 5 represent the kinetics of the synthesis of the complementary DNA strand and of the appearance of the Fok I fragments, as outlined in Figs. 1 and 2. M13mp7 ss DNA (2 μg) was suspended in 4 μl of 10 m*M* tris-HCl + 1 m*M* EDTA (*p*H 7.5); 10 μl of 10× Fok I buffer were added, together with a 7.5× molar excess of the 34-mer adapter-primer (Fig. 1). Water was added (to attain the final reaction volume of 100 μl), and the sample was heated for 10 minutes at 70°C and then annealed for 1 hour at 23°C. PolIk [10 units; New England Biolabs (*8*)] and all four dNTPs (8 μl of 2.5 m*M*) were added, and the reaction was carried out for 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4), or 2 hours (lane 5). Fok I (8 units) was added 5 minutes after the addition of PolIk. The reaction was stopped by adding 100 μl of 5*M* ammonium acetate and 400 μl of 95% ethanol. Samples were placed in dry ice; the precipitate was spun in a microfuge, washed twice with 70% ethanol, dried, redissolved in Fok I buffer, separated by electrophoresis in a 1.5% agarose gel, and stained with ethidium bromide. The control samples in lanes 1, 6, and 7 were run in the same gel. The sample in lane 1 was treated in the same manner as in lane 4, but no 34-mer adapter-primer was added. Fragment sizes are specified in base pairs on the right margin and correspond to those shown in Fig. 2.

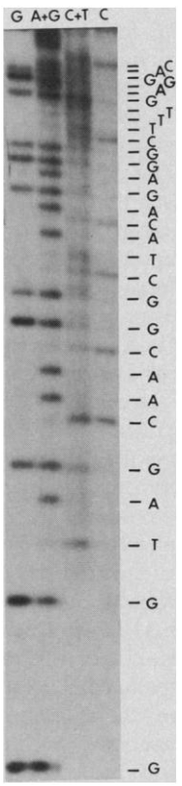

Fig. 4. Nucleotide sequence of the Fok I adapter-primer–induced fragment. The 1116-bp fragment (actually 1112 bp with two 4-nt extensions; 1120 bp upon PolIk–directed fill-in) was isolated from a 1.5% agarose gel, identical to that shown in Fig. 3, lane 2 or 3. The isolated fragments were treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) to remove 5′ phosphate, and the 5′-OH ends were labeled with [γ-^{32}P]adenosine triphosphate and T4 polynucleotide kinase (*8*). The end-labeled fragment was cleaved with Hin PI (*8*), and the fragment with the adapter-primer–induced cut was isolated on a 5% polyacrylamide gel and sequenced by the chemical cleavage method (*13*). The lanes are marked G, A+G, C+T, and C for the four cleavage reactions, and the deciphered sequence is shown on the right margin starting with the 5′-terminal G at the bottom (compare with the sequence in Fig. 1B, right fragment, upper strand). The sequence was determined by means of a 20% polyacrylamide sequencing gel and the Maxam and Gilbert procedure (*13*) with the following modifications: we removed piperidine after all chemical cleavage reactions by transferring the reaction mixture to a clean plastic microtube and carrying out five cycles of lyophilization and dissolving in 50 μl of water. Moreover, the gel electrophoresis was stopped when the blue dye marker reached only two-thirds of the gel.

with a mixture of DNA fragments that cause nonspecific priming.

The experimental approach we describe is not limited to Fok I. The procedure probably could be adapted to over 15 other class-IIS enzymes, including Bbv I, Bbv II, Bin I, Bsp MI, Eco 31I, Eco 57I, Gsu I, Hga I, Hph I, Mbo II, Mme I, Mnl I, Sfa NI, Taq II, Tth 111II, and others (*1, 2*). In the experiments of Podhajska and Szybalski (*3*) the class-IIS restriction enzymes had to be free of endonucleolytic activity directed toward ss DNA; this requirement is of much less importance for the ds DNA digestion experiments described here. Our method created one predetermined end, whereas the other terminus could be produced by a cut with any other enzyme, not only by Fok I, as described here.

Theoretically, two more improvements could have been made in our method. Replacement of deoxycytidine 5′-triphosphate (dCTP) by 5-methyl-dCTP during the PolIk-mediated DNA synthesis (Fig. 1) might have eliminated all other Fok I cuts on the RF molecule (Fig. 2). However, methylation of the C residues has little or no effect on Fok I recognition or cleavage functions (*9*). Alternatively, the Fok I sites originally present in M13mp7 DNA could perhaps be inactivated by M·Fok I methylase (*8*); however, this might not be practical because our original target is an ss DNA molecule and, moreover, Pósfai and Szybalski (*9*) have discovered that M·Fok I methylates in an asymmetric manner only the unique A located on one of the strands (GGATG) of the Fok I recognition site.

In conclusion, we have designed and described a four-component system which, by the use of a specially designed adapter-primer oligodeoxynucleotide, permits one to produce DNA fragments with a predetermined end located between any two base pairs of the target DNA. This approach is especially useful for DNA fragments cloned in ss DNA-generating vectors (*10*), and it could also complement our other preprogrammed DNA trimming method (*11*). The present experiments should also be important for dissecting the mechanisms of enzyme recognition, endonucleolytic cleavage, and methylation (*9, 12*).

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Stimulation of RNA and Protein Synthesis by Intracellular Insulin

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Like insulin-sensitive somatic cells, stage IV oocytes from *Xenopus laevis* increase their synthesis of RNA, protein, and glycogen in response to extracellular insulin. Synthesis of RNA and protein are also increased when oocytes are maintained under paraffin oil and insulin is microinjected into the cytoplasm. The effects of external and intracellular insulin are additive, suggesting separate mechanisms of action. Experiments with nuclei isolated under oil show that RNA synthesis can be stimulated by applying insulin to the nucleus directly. Thus, the nucleus appears to be one intracellular site of hormone action.

IN SENSITIVE CELLS, INSULIN REGULATES many activities, including membrane transport, energy metabolism, transcription, and translation. Evidence points to the plasma membrane as a primary site of action for the rapid effects of insulin. However, the detailed mechanism (or mechanisms) by which insulin alters cytoplasmic and nuclear function over longer intervals remains to be established. Researchers have focused on potential second messengers (*1*) and signaling through protein phosphorylation (*2*), but other mechanisms could be involved. One possibility is that the insulin molecule plays a direct role in intracellular regulation, but evidence for this is circumstantial. (i) Cells take up insulin from the extracellular medium (*3*); the fate of internalized hormone (with or without receptor) appears to be dependent on cell type (*4*). (ii) Insulin receptors are found inside many cells (*5*); the function of these receptors is unknown. (iii) Experiments with isolated nuclei suggest that the nuclear envelope is one site of insulin action. The envelope contains specific receptors, and insulin exposure increases envelope phosphorylation, RNA release to the medium, and envelope pore permeability to dextrans (*6, 7*).

Testing the hypothesis that intracellular insulin controls metabolic activity requires an experimental system in which hormone can be introduced into a cell with minimal disruption and metabolic flux can be measured in the same cell. Technical problems related to small cell size make such experiments impossible with most insulin-sensitive cells. My approach was to use a giant cell, the *Xenopus laevis* oocyte, which can be studied by the use of intracellular microinjection techniques and single cell analysis.

Research on mechanisms of insulin action

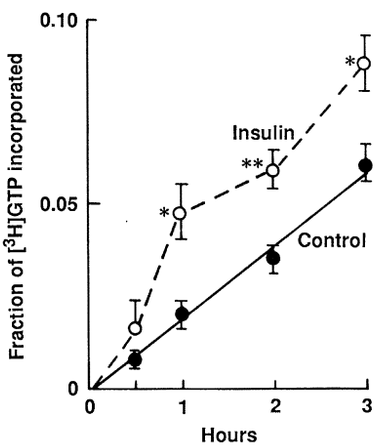

Fig. 1. Cells were transferred to oil and microinjected with 25 nl of intracellular buffer containing 0.01% BSA, 450 fmol of ^{3}H-labeled GTP, and 0 or 18 ± 2 fmol of insulin. After 0.5 to 3 hours, cells were processed to determine GTP incorporation, as described in Table 1. Each point represents the mean fraction incorporated from 7 to 12 (control) or 4 to 6 (insulin) oocytes; variability is given as SE bars. Statistical comparisons by protected *t* test (*$P < 0.05$; **$P < 0.01$).

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