Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1082-1088

BACTERIAL CLONING OF THE RABBIT UTEROGLOBIN STRUCTURAL GENE

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Received March 7,1980

SUMMARY

Double-stranded DNA complementary to partially purified uteroglobin messenger RNA was synthesized using reverse transcriptase. Integration into the pBR 322 plasmid was accomplished by the oligo dG-dC tailing procedure. Bacterial clones were selected by hybridization with (^{32}P) DNA complementary to uteroglobin mRNA. After amplification, the integrated DNA was isolated by Pst I digestion and was shown by partial sequencing to contain regions coding for uteroglobin.

INTRODUCTION

Rabbit endometrium has been widely used to test the biological activity of progestative hormones (1). To elucidate the mechanisms of action of a steroid hormone at the molecular level, it is necessary to follow the induction of a specific protein and of its messenger RNA. Uteroglobin (2), also called blastokinin (3), is a good subject for such studies since it is a protein secreted in large quantities by the rabbit endometrium under the influence of progesterone. The messenger RNA for uteroglobin has been translated (4-6) and purified (4). A complementary DNA has been obtained (7,8). In this report, we describe the cloning of this cDNA in E. coli.

MATERIALS AND METHODS

1. Enzymes

Reverse transcriptase was the kind gift of Drs J.W. Beard and M.A. Chirigos [Office of Program Resources and Logistics. Viral Cancer Program. Viral Oncology. Division of Cancer Cause and Prevention. National Cancer Institute (Bethesda, M.D. 20014)]. Pst I and Alu endonucleases I were obtained from Biolabs, (Beverly, Ma.). Terminal tranferase and S_1 nuclease were a gift of Dr. F. Rougeon (Institut Pasteur, Paris).

0006-291X/80/081082-07$01.00/0

2. Synthesis of double stranded cDNA

The messenger RNA (6 µg) prepared as previously described (4), was incubated for 50 min. at 42°C with 50 units of reverse transcriptase in 250 µl of Tris-HCl 50 mM (pH 8.2) buffer containing KCl 75 mM, dithiothreitol 10 mM, $MgCl_2$ 10 mM, (α^{32}P) dCTP 0.125 mM, dGTP, dATP, dTTP 0.25 mM, sodium pyrophosphate 4 mM, oligo dT_{12-18} 5 µg/ml. cDNA synthesis was terminated by cooling the incubation mixture and adding 10 µl of a 0.25 M solution of EDTA. RNA was hydrolyzed by incubation for 2 h. at 50°C in 0.3 M NaOH. After neutralization, the cDNA was chromatographed on Sephadex G-75. Fractions eluting in the void volume were ethanol-precipitated and used as a template for the synthesis of double-stranded cDNA. This double-stranded cDNA was prepared by incubation for 2 h. at 37°C under the same conditions as above but in the absence of oligo-dT and pyrophosphate. Non-radioactive dCTP was used at a 0.25 mM concentration. The total volume of incubation was 200 µl. Incubation was terminated by cooling and addition of EDTA to a final concentration of 10 mM.

After extraction with phenol-chloroform (1 : 1 volume/volume) the cDNA was chromatographed on Sephadex G 75 and the void volume fractions precipitated with ethanol. S_1 nuclease digestion was carried out for 60 min. at 37°C in 1 ml of a sodium acetate 50 mM (pH 4.5) buffer containing zinc sulfate 1 mM, potassium chloride 300 mM and glycerol 5%. For 1.8 µg of cDNA 9 units of S_1 nuclease were used. After extraction by phenol-chloroform, the double-stranded cDNA was ethanol-precipitated and centrifuged 5 h. at 20°C on a 5-20% sucrose gradient in Tris 0.01 M (pH 7.4) buffer containing EDTA 1 mM, NaCl 1 M (rotor SW 50.1, 49,000 rpm). Fractions corresponding to the faster-migrating two-thirds of the radioactive peak were recovered and ethanol-precipitated.

3. Elongation of the double-stranded cDNA and of the pBR322 plasmid and transformation of E. coli.

Double-stranded cDNA (50 ng) was elongated by terminal transferase (6 units) in 100 µl of potassium cacodylate buffer 200 mM (pH 6.7), containing cobalt chloride 1 mM, β-mercaptoethanol 0.1 mM and dCTP 0.1 mM. The incubation period was 4 min. at 35°C. The reaction was stopped by bringing the mixture to 10 mM EDTA and 0.2% sodium dodecylsulfate. Elongated cDNA was ethanol-precipitated.

pBR 322 plasmid (2 µg) was made linear by digestion with Pst I (incubation for 2 h. at 37°C with 2.4 units) under the conditions described by the manufacturer. The plasmid was ethanol-precipitated and elongated as described above, with the following exceptions : dGTP was used instead of dCTP, $MgCl_2$ 8 mM replaced $CoCl_2$, β-mercaptoethanol was 2 mM and incubation was for 20 min. at 35°C. The elongated plasmid (0.1 µg) was ethanol-precipitated and annealed to the dC elongated cDNA (25 ng) for 10 min. at 65°C in 100 µl of Tris HCl 20 mM (pH 8.1) buffer containing NaCl 0.3 M, EDTA 1mM. The incubation mixture was cooled gradually to 25°C. The chimeric plasmid was used to transform K 12 Escherichia coli (DP 50 sup F strain) (9). Clones containing recombinant plasmids were isolated by virtue of their resistance to tetracycline and identified initially by their sensitivity to ampicillin.

4. Detection and amplification of hybrids containing sequences complementary to uteroglobin mRNA.

(^{32}P) cDNA was synthesized from uteroglobin mRNA (see above). This cDNA was further purified by molecular hybridization

[(7) and (M. Atger, J.F. Savouret, E. Milgrom, submitted for publication)] to isolate the 30% most abundant copies which have been shown to constitute a homogenous population corresponding to uteroglobin messenger RNA. E. coli colonies containing hybrid plasmids were transferred onto nitrocellulose filters, lyzed and the DNA denatured. Hybridization with (^{32}P) uteroglobin cDNA was carried out for 16 h. at 68°C as described (10) with 400,000 cpm per filter (diameter 8.5 cm). After washing of the filters, radioactive colonies were detected by autoradiography. For the colonies showing the highest levels of radioactivity, the plasmids were amplified, bacteria lyzed and plasmid DNA isolated (11).

5. DNA sequencing was performed by the method of Maxam and Gilbert (12). Each determination was carried out 4 times.

6. Biohazards.

Possible biohazards associated with the experiments described in this publication were examined by the French National Control Committee. Experiments were conducted under L_3 B_1 conditions.

RESULTS

1. Construction and amplification of hybrid plasmids

A preparation of messenger RNA, one-third of whose sequences corresponded to uteroglobin (4), was utilized. Single-stranded cDNA was obtained in high yield using the method of Myers and Spiegelman (13) as modified by F. Rougeon (personnal communication) : 6 µg of mRNA gave 4.9 µg of cDNA.

The yield of double-stranded cDNA was lower : 0.45 µg being obtained with 1.4 µg of single-stranded cDNA. One hundred ng of dG-elongated pBR 322 was annealed with 25 ng of dC-elongated double-stranded cDNA and the product used to transform the bacteria. This gave 159 colonies resistant to tetracycline and sensitive to ampicillin.

Hybridization with purified (^{32}P) uteroglobin cDNA revealed 50 radioactive spots, thus confirming that the messenger RNA preparation which was used contained about one-third sequences corresponding to uteroglobin. Twenty-four of the colonies gave very strong signals on hybridization in situ. Eighteen of these colonies were amplified and their DNA was purified (11).

2. Characterization of the inserted sequences

The DNA from the hybrid plasmids was digested by Pst I and analyzed by polyacrylamide gel electrophoresis (Fig. 1). In all cases a single DNA fragment was observed in addition to the pBR 322 plasmid. This demonstrated the absence of Pst I sites inside

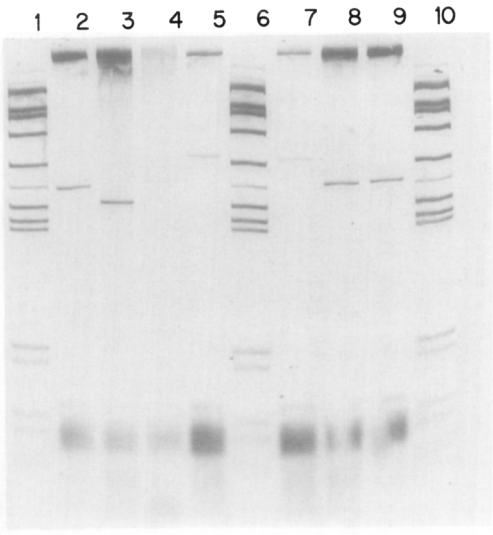

Fig. 1 : Electrophoretic analysis of inserted DNA in hybrid plasmids.
DNA, from plasmids shown by molecular hybridization to contain uteroglobin mRNA sequences, was treated with Pst I and analyzed by polyacrylamide (8%) gel electro phoresis. Lines 1, 6 and 10 show migration of markers (910, 659, 655, 521, 403, 281 and 257 base pairs etc...) obtained by digestion of pBR 322 by Alu I. Clones UGL 3 (line 2), UGL 4 (line 3), UGL 11 (line 4), UGL 12 (line 5), UGL 13 (line 13), UGL 14 (line 8) and UGL 15 (line 9) are also shown.

the inserted sequences. Two of the inserts were larger than 400 base-pairs (435 and 455 respectively) ; seven were of a size between 300 and 400 base-pairs.

3. Partial sequencing of the inserted DNA

Sequencing studies were performed on clone UGL 15 (350 base-pairs long). Preliminary experiments indicated that the inserted sequence was cleaved by Alu I into two fragments, one of 230 and one of 120 base-pairs. These fragments were partially sequenced in order to identify without ambiguity the inserted DNA as corresponding to uteroglobin mRNA (Table 1). In the smaller fragment 60 nucleotides were identified. These corresponded to aminoacids 32-51 of uteroglobin. Identification of nucleotides in the gel started approximately 5 nucleotides behind the migration line of Xylene Cyanol. This marker migrates on electrophoresis as a fragment of about 35 base-pairs. Thus the smaller fragment extends about 40 base-pairs in the 5' direction. The last nucleotides of this fragment should thus correspond approximately to aminoacid 19.

TABLE 1

Nucleotide sequences in the inserted DNA of plasmid UGL 15

1) Small fragment obtained after Alu I digestion.

Observed nucleotides	CTG TGG TAC TTT CTA CGT CCC TAC GTC TAC TTC TTC CAC AAC
Complementary nucleotides 5' ←	GAC ACC ATG AAA GAT GCA GGG ATG CAG ATG AAG AAG GTG TTG
Aminoacid (number in	Asp Thr Met Lys Asp Ala Gly Met Glu Met Lys Lys Val Leu
sequence of the protein)	(32)(33)(34)(35)(36)(37)(38)(39)(40)(41)(42)(43)(44)(45)
	TTG TGG GAT GGG GTT TGA..
	AAC ACC CTA CCC CAA ACT → 3'
	Asn Ser Leu Pro Gln Thr
	(46)(47)(48)(49)(50)(51)

2) Large fragment obtained after Alu I digestion.

Observed nucleotides ...	CTCAATAAACGCAGCAGATCAAAAAAAAAAAAAAAAA....
5' ←	→ 3'

DNA from clone UGL 15 was treated with Pst I, dephosphorylated and labeled with (γ ^{32}P)ATP. The inserted fragment was recovered and digested into two fragments using Alu I. Partial sequences of these fragments are shown.

The larger fragment started with dC residues corresponding to the region of double-stranded cDNA elongated by terminal transferase. The next 35 nucleotides were identified : 17 were dA, followed by a non-coding sequence of 18 nucleotides. The larger Alu I fragment thus corresponds to the 3' end of the messenger. The region for which the sequence of deoxynucleotides has been determined does not code for the protein. At positions 12-17 after poly A a sequence of AATAAA is found which has previously been described to be in the 3' non coding regions of various messengers (14).

4. Construction of plasmids containing "full length" cDNA.

We have recently prepared new hybrid plasmids. Using clone UGL 15 as a probe, we have identified 50 plasmids containing uteroglobin cDNA sequences. One of them has a length of 575 base-pairs and thus contains the entire or almost the entire uteroglobin cDNA (messenger size $\sim$ 600 nucleotides).

DISCUSSION

Hybrid plasmids were constructed using double-stranded cDNA obtained from a RNA preparation containing uteroglobin messenger RNA. Inserted sequences were identified by hybridization with (^{32}P) uteroglobin cDNA and by sequencing.

Since sequencing studies were aimed primarily at identifying the inserted DNA, a plasmid of 355 nucleotides (clone UGL 15) was selected to increase the probability of observing nucleotides coding for the protein at either end [The aminoacid sequence of uteroglobin is known (15,16)]. This was, in fact, achieved, since 20 aminoacids corresponding to positions 32-51 were identified. The previously published aminoacid sequence was thus confirmed with a single exception : the aminoacid residue 46 given as Asp (15) or Asx (16) is shown here actually to be Asn.

The inserted sequence in clone UGL 15 extends from the 3' end up to the region corresponding approximately to aminoacid 19. It lacks nucleotides coding for the remaining 39 aminoacids (including 21 aminoacids of the signal peptide) and nucleotides for the 5' non-coding region. The largest inserted sequence (575 base-pairs) should contain all (or almost all) of the nucleotides represented in uteroglobin messenger RNA.

We are presently trying to detect, in a library of rabbit chromosomal fragments (17), DNA sequences hybridizing to uteroglo

bin structural gene. Studies of progesterone receptor interaction with sequences present in the genomic clone(s) would be of interest.

ACKNOWLEDGEMENTS

We thank Dr. F. Rougeon (Institut Pasteur) for communicating unpublished results.

REFERENCES

1. Goldman, B. and Zarrow, M.X. (1973) in Endocrinology, Handbook of Physiology, (Greep R.O. and Astwood, E.B. Eds) II, 547-572, Williams & Wilkins Company, Baltimore.
2. Beier, H.M. (1968) Biochim. Biophys. Acta 160, 289-291.
3. Krishnan, R.S. and Daniel, J.C. (1967) Science 158, 490-492.
4. Atger, M. and Milgrom, E. (1977) J. Biol. Chem. 252, 5412-5418.
5. Beato, M. and Nieto, A. (1976) Eur. J. Biochem. 64, 15-25.
6. Bullock, D.W., Woo, S.C. and O'Malley, B.W. (1976) Biol. Reprod. 14, 194-201.
7. Atger, M., Mornon, J.P., Savouret, J.F., Loosfelt, H., Fridlansky, F. and Milgrom, E. (1979) , in press, Elsevier/North Holland Biomedical Press, Amsterdam.
8. Arnemann, J., Heins, B. and Beato, M. (1979) Eur. J. Biochem. 99, 361-367.
9. Leder, P.T., Tiemeier, D. and Enquist, L. (1977) Science 196, 175.
10. Grunstein, M. and Hogness, M.S. (1975) Proc. Natl. Acad. Sci. USA 72, 3961-3965.
11. Tanaka, T. and Weisblum, B. (1975) J. Bacteriol. 121, 354-362.
12. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
13. Myers, J.C. and Spiegelman, S. (1978) Proc. Natl. Acad. Sci. USA 75, 5329-5333.
14. Proudfoot, N.J. and Brownlee, G.G. (1976) Nature 263, 211-214.
15. Ponstingl, H., Nieto, A. and Beato, M. (1978) Biochemistry 17, 3908-3912.
16. Atger, M., Mercier, J.C., Haze, G., Fridlansky, F. and Milgrom, E. (1979) Biochem. J. 177, 985-988.
17. Maniatis, J., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. and Efstratiadis, A. (1978) Cell 15, 687-701.