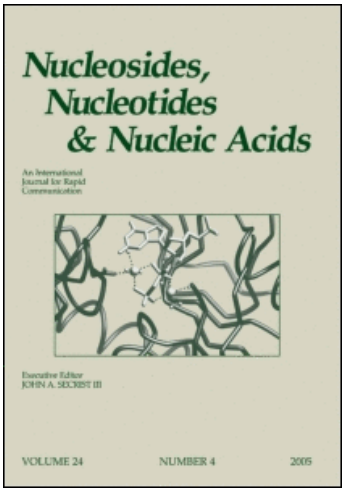

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Sachse, Hans , Hagendorff, Gerd , Preuß, Klaus Dieter , Sharma, Hari S. and Scheit, Karl Heinz(1988) 'Synthesis, Molecular Cloning and Expression of Genes Coding for Atrial Natriuretic Factors from Rat and Human', Nucleosides, Nucleotides and Nucleic Acids, 7: 1, 61 — 73
To link to this Article: DOI: 10.1080/07328318808068703
URL: http://dx.doi.org/10.1080/07328318808068703

NUCLEOSIDES & NUCLEOTIDES, 7(1), 61-73 (1988)

SYNTHESIS, MOLECULAR CLONING AND EXPRESSION OF GENES CODING FOR ATRIAL NATRIURETIC FACTORS FROM RAT AND HUMAN

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Abstract: Genes coding for the amino acid sequence 1-33 of atrial natriuretic factor from rat as well as human were synthesised, cloned into the C-terminal part of the ß-galactosidase gene of plasmid pUR289. The derived plasmids prANF1 and phANF1 were characterised by restriction analysis as well as sequencing and expressed in E.coli. Gene expression after induction led to formation of fusion proteins, which were found to contain C-terminal peptides sequences attached to ß-galactosidase which were immuno reactive with antiserum against human atrial natriuretic factor.

INTRODUCTION

Chemical synthesis, molecular cloning as well as expression of a number of genes for small proteins has been described (1,2,3,4). Efficient gene expression and production of the foreign protein in E.coli appeared to be difficult due to the endogenous proteolytic activities of the host cells. To overcome this difficulty, the synthetic DNA genes were fused to the C-terminal part of the lacZ gene resulting in the expression of hybrid proteins of large molecular weight. Due to the altered polypeptide sequence of the ß-galactosidase portion of the fusion protein, they tend to aggregate and thereby becoming insoluble when overproduction of the fusion protein is induced by isopropyl-ß-D-thiogalactoside (IPTG) (1,5). The main aim for the construction and expression of hybrid genes was to

achieve the isolation of the attached smaller protein fragment after cleavage.

It was reported for a number of fusion proteins that the attached small protein fragment was still recognized by an antiserum raised against the small protein itself (1,3). This implied that those fusion proteins should also elicit antibodies in animals with the specificity for the small protein part. Furthermore, one should keep in mind that most antisera against small proteins, like hormones, were obtained after conjugating the small protein to a larger carrier protein to increase antigenicity. Often the small proteins are not easy to purify; in the case of hormones the quantities of homogenous proteins necessary for immunization are difficult to obtain. Therefore, we decided to construct hybrid genes coding for two bioactive peptides, atrial natriuretic factor from rat and human to obtain the respective fusion proteins in order to explore their potential use as antigens for the production of the corresponding antisera.

The mammalian cardiac atrium has recently been shown to contain factors which induce potent natriuresis (6,7), changes in renal haemodynamics (8) and relax pre-contracted vascular smooth muscles (9). These factors, collectively called atrial natriuretic factor (ANF), are derived from larger precursor molecules. In rat, the circulating form of rANF is comprised of 28 amino acids, whereas the circulating form of hANF is not yet known. Saidah et al. (10) reported the amino acid sequence of rANF,isolated from rat atria, consisting of 33 amino acids. This sequence was supported by the cloned cDNA for the rANF precursor (11,12). Similarly, the sequence of the hANF precursor cDNA was reported (13,14). We decided to synthesise genes coding for rANF of 33 amino acids and hANF of 32 amino acid residues in accord with the published cDNA sequences (10,13,14).

The synthetic ANF genes will be fused to the ß-galactosidase gene of E.coli (lacZ) with the lacZ gene with the final aim to obtain the respective fusion proteins for immunochemical studies.

MATERIAL AND METHODS

Oligonucleotide synthesis

Oligonucleotides were synthesised by means of a Model 381A DNA-synthesiser from Applied Biosystems utilising phosphoramidite chemistry and after deprotection, purified by polyacrylamide gel elecectrophoresis in 7M urea.

Bacterial strains and plasmids

pUR289 as well as **E.coli** BMH71-18 (15) were kindly provided by B.Müller-Hill. BMH71-18/pUR289 was grown in LB-medium containing 100 µg/ml ampicillin.

DNA sequencing

EcoRI fragments obtained from prANF1 or phANF1 respectively were subcloned into M13mp8(RF). Derived M13 single stranded DNA was sequenced according to Sanger et al. (16).

Construction of chimeric expression plasmids

Each oligonucleotide (1.34 pmol) was phosphorylated separately in a buffer containing 70 mM Tris-HCl,pH7.6, 10 mM $MgCl_2$, 0.1 mM EDTA, 40 pM ATP and 6 units T4-polynucleotide kinase. After incubation for 30 min at 37°C, the reaction mixtures were heated for 5 min at 100°C. All reaction mixtures were combined and centrifuged through Sephadex G 50 (medium) in a blue Eppendorf pipet tip. The oligonucleotides were precipitated at a concentration of 0.3 M sodium acetate (pH 5.2) by addition of ethanol. After chilling, the precipitates were collected by centrifugation; the pellets were washed with 80% ethanol, dried and dissolved in 100 µl water. The vector pUR289 was cut with BamHI and dephosphorylated by alkaline phosphatase following a standard protocol (17). The mixture of phosphorylated oligonucleotides (67 fmoles each) in buffer containing 50 mM Tris-HCl,pH7.6, 0.1 mM EDTA, 0.1 mM spermidine, 10 mM $MgCl_2$, 1 mM ATP and 4.5 mM DTT was kept for 5 min at 37°C and allowed to slowly cool to 15°C. Then 30 fmol of pUR289, treated as described above, as well as 4 units of T4-ligase were added and incubation continued for 15h at 15°C. The ligation mixtures were directly employed to transform **E.coli** BMH71-18 using standard conditions (17). Bacterial colonies, growing on LB-agar containing 100 µg/ml ampicillin, were screened by restriction analysis of plasmid DNA isolated from cells by a standard

procedure. EcoRI inserts of approximately 220 bp, in comparison to those of 101 bp derived from pUR289 were isolated and subjected to sequencing.

Preparation of crude extracts

Cells from a 10 ml culture, collected by centrifugation, were suspended in 1 ml homogenisation buffer containing 50mM Tris-HCl, pH 7.9, 2 mM EDTA, 1 mM mercaptoethanol, 235 mM NaCl, 5% (w/v) glycerol, 30 µg/ml phenylmethylsulfonyl fluoride and 375 µg/ml lysozyme. The mixture was kept on ice for 20 min followed by addition of 500 µg/ml sodium deoxycholate and incubation continued on ice for 30 min. The suspension was then homogenised for 1 min in ice by means of an ultraturrax (Jahnke & Kunkel,Staufen). After centrifugation at 8000 rpm at 4°C for 20 min, supernatant (crude extract) and pellet were separated and saved. The pellet was dissolved in 300 µl of 2% (w/v) SDS, 0.5M Tris-HCl, pH 6.8, 5% (w/v) glycerol and 5% (w/v) mercaptoethanol and the resulting mixture kept at 100°C for 5 min.

Gel elecctrophoresis and immuno-blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (18) and immunoblotting (19) after SDS-PAGE followed a published protocol. For immuno-detection of rANF and hANF the blots were incubated with sheep anti-hANF antiserum (Fa. IBL, Hamburg) at a dilution of 1:1000 and peroxidase-conjugated anti-sheep IgG antibodies (Amersham) at a dilution of 1:1000. Both antiserum dilutions were supplemented with crude soluble proteins from **E.coli** to a concentration of 10 µg/ml and the mixtures kept for 1 h at 25°C prior to use.

RESULTS AND DISCUSSION

The known amino acid sequences for rANF as well as hANF were translated into a DNA sequence employing the codon usage in highly expressed **E.coli** genes (20). In the past the cleavage of fusion proteins by CNBr at methionine, not contained in the small protein fragment, was a useful approach to generate the desired small polypeptides (1,2). Because we were principally also interested in a specific cleavage of the expressed fusion protein with the aim of obtaining the peptide fragment corresponding to the synthetic

DNA-fragment, we had to decide by which way the fusion between lacZ gene and synthetic DNA could be accomplished. rANF did not contain methionine, therefore a CNBr-susceptible methionine link between ß-galactosidase and the small peptide fragment would be feasible. However, the amino acid sequence of hANF, in contrast to rANF, differed by the presence of a methionine instead of an isoleucine and therefore this strategy could not be followed. Recently Nagai and Thorgersen described the construction of a hybrid gene containing the human ß-globin gene and part of the λ-cII gene (21). The sequence which linked the two DNA moieties represented a DNA sequence coding for a tetrapeptide sequence which was the target for a specific protease, the blood coagulation factor Xa. The target sequence selected by those authors was IEGR. We adopted this strategy for our purpose and inserted into the 5'-part of the synthetic DNAs for rANF a dodecanucleotide sequence coding for the amino acids IEGR. In the case of hANF the amino acid isoleucine in the target sequence for factor Xa was replaced by leucine. This change created a BamHI site which would be regenerated after cloning. Since isoleucine in natural substrates can also be replaced by alanine (22), the exchange isoleucine<->leucine appeared justified.

The double stranded DNA sequences coding for rANF and hANF are shown in Figure 1. The sequence at the N-terminal part was extended by the information for the four amino acids constituting the target sequence for factor Xa. To achieve termination, two stop codons were introduced at the 3' end of the sense strand. The double stranded DNA fragment contained at the 5'-ends BamHI sequences for insertion into a vector. In the case of the synthetic DNA for rANF only the BamHI at the 3'-end, in the case of hANF both BamHI sites would be regenerated after cloning. The DNA for rANF as well as hANF was dissected into four complementary sequences respectively which provided 10 base pair overlaps for ligation to join the sequences to the complete DNA.

We chose pUR289 (23) as an expression vector, which contains a suitable polylinker region for the insertion of synthetic DNA fragments into the C-terminal region of lacZ gene (Fig. 2). The resulting fusion proteins should consist of 1024 amino acid residues from ß-galactosidase and 37 or 36 amino acids in the case of rANF and hANF respectively.

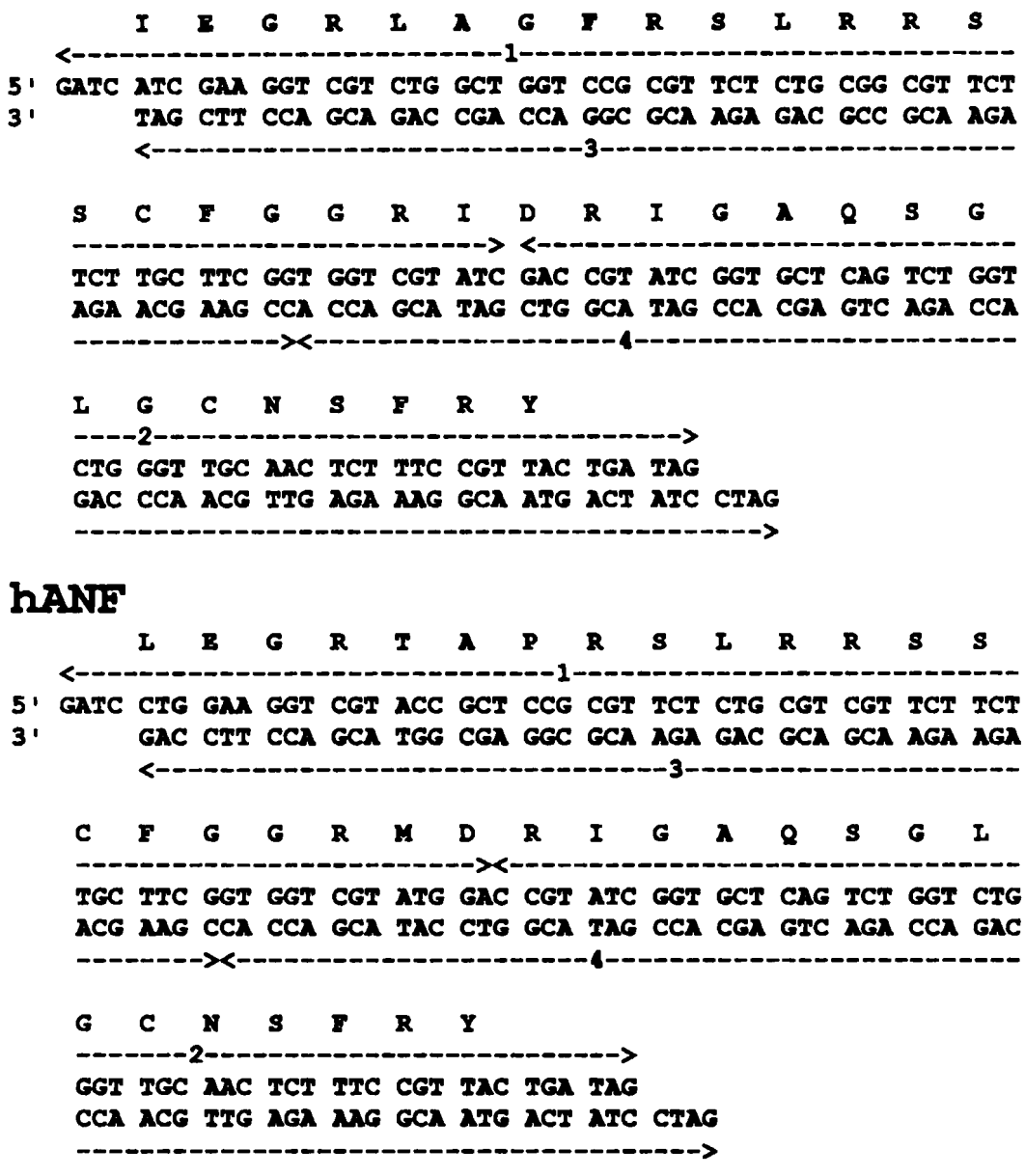

FIGURE 1 Synthetic DNA coding for rANF 1-33 and hANF 1-33
The numbered sequences marked by dashes represent the chemically synthesised deoxyoligonucleotides.

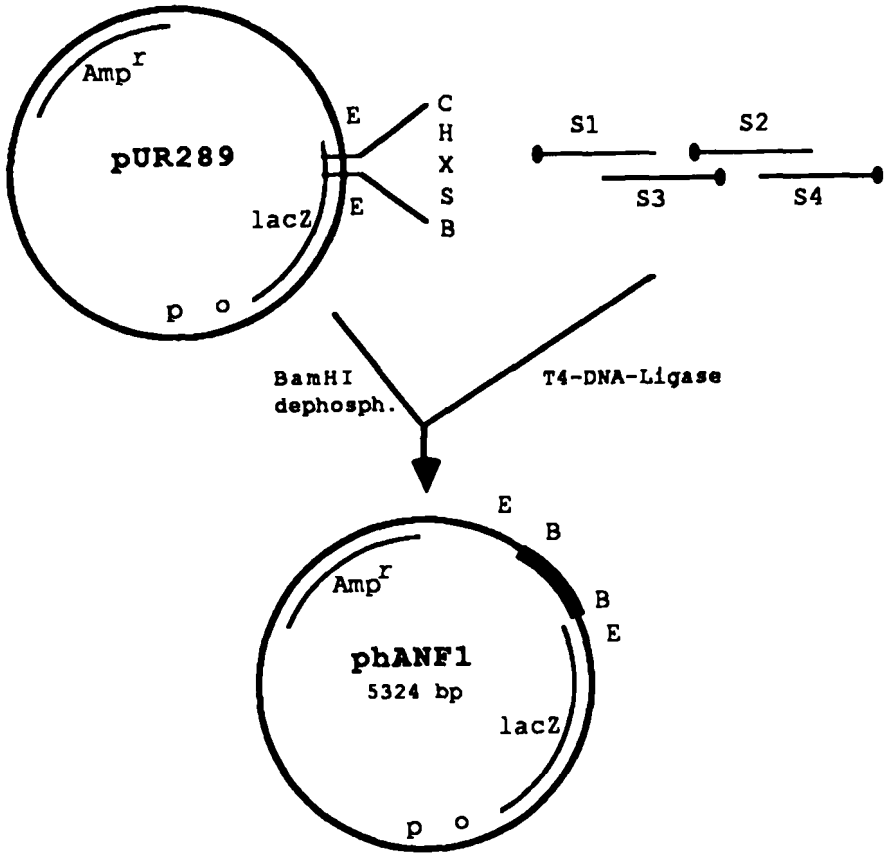

FIGURE 2 Construction of phANF1
The dots at the deoxyoligonucleotide symbols S1-S4 represent 5'-phosphate residues. Abbreviations: E,EcoR1; B,BamH1; S,Sal1; X,Xba1; H,Hind3, C, Cla1.

The vector pUR289 was restricted with enzyme BamHI, dephosphorylated and ligation performed in the presence of a mixture of the respective synthetic oligonucleotides which were phosphorylated at their 5'ends (Fig. 2). The ligation reaction mixtures were employed to transform the E.coli strain BMH71-18. Positive transformants were detected by the following screening protocol: plasmids were isolated from colonies and restricted with EcoRI; the EcoRI-fragments of plasmids from positive colonies had a larger size compared to the EcoRI-insert of pUR289 with 101 bp. Due to the fact that the synthetic DNA fragments possessed symmetrical restriction

EcorRI insert of prANF1

AATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAGGG**GATCATCGAAGGTC**
GTCTGGCTGGTCCGCGTTCTCTGCGGCGTTCTTCTTGCTTCGGTGGTCGTATCGACCG
TATCGGTGCTCAGTCTGGTCTGGGTTGCAACTCTTTCCGTTACTGATAGATCCGTCGAC
TCTAGAAAGCTTATCGATGATAAGCTGTCAAACATGAGAATTC

EcoRI insert of phANF1

AATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAGGG**GATCCTGGAAGGTC**
GTACCGCTCCGCGTTCTCTGCGTCGTTCTTCTTGCTTCGGTCGTATGGACCGTATCGG
TGCTCAGTCTGGTCTGGGTTGCAACTCTTTCCGTTACTGATAGGATCCGTCGACTCTAGA
AAGCTTATCGATGATAAGCTGTCAAACATGAGAATTC

FIGURE 3 DNA sequences of EcoR1 inserts of prANF1 and phANF2
The nucleotides in bold print represent the synthetic BamH1 fragment. Insert size: prANF1, 222; phANF1, 219.

sites, two orientations of ligation into pUR289 were to be expected. Therefore the EcoRI-fragments were subcloned into M13mp8(RF) and the sequences were determined employing the dideoxy method of Sanger et al. (16). The EcoRI-fragments possessing the correct sequences of the synthetic DNA's and in proper orientation led to the identification of positive colonies harbouring the desired plasmid. The corresponding plasmids were designated prANF1 and phANF1. The sequences of the corresponding EcoR1 inserts are given in Figure 3.

Restriction analysis of the EcoRI-fragments of the above plasmids should yielded the following fragments: prANF1, EcoR1/Ava2->147+75; phANF1, EcoR1/Ava2->116+103. Experimental results proved that this was true (data not shown).

Expression of prANF1 and phANF1

Exponentially growing cultures of BMH71-18/prANF1 and BMH71-18/phANF1 were induced by addition of IPTG to a final concentration of 0.5mM. Crude extracts of soluble proteins were prepared after induction and subjected to SDS-PAGE (Figs. 4,5). In accord with the findings by other authors (1,2,3) most of the fusion protein was found associated with insoluble material. Therefore, at various times after induction,

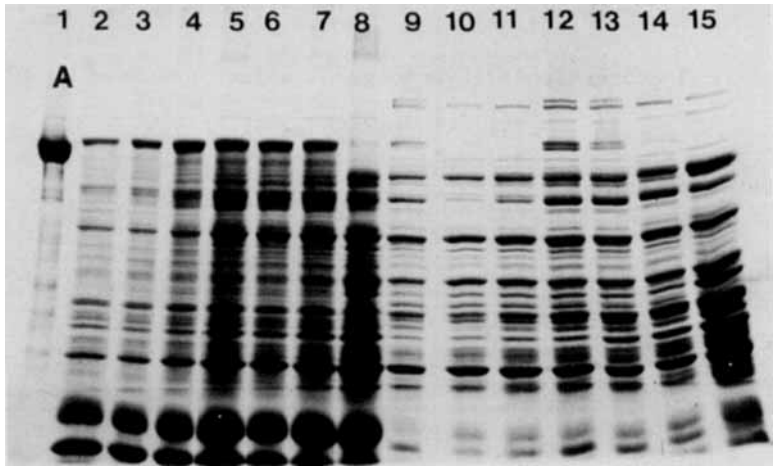

FIGURE 4 SDS-PAGE of proteins extracted from BMH71-18/prANF1 after induction by IPTG
Lane 1, ß-agalactosidase, 10 µg; lanes 2-8, insoluble proteins 1h20',2h10',3h,4h,4h45',6h10' and 18h after induction; lanes 9-15, soluble proteins 1h20',2h10',3h,4h,4h45',6h10' and 18h after induction respectively.

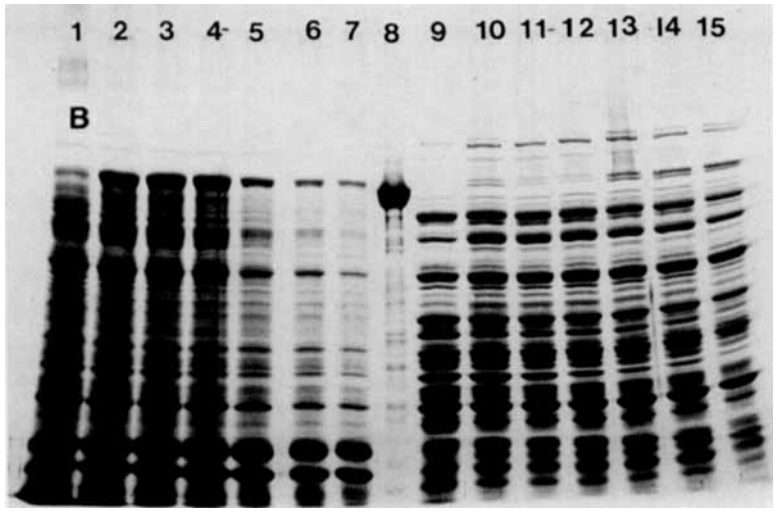

FIGURE 5 SDS-PAGE of proteins extracted from BMH71-18/phANF1 after induction by IPTG
Lanes 1-7, insoluble proteins, 18h,6h10',4h45',4h,3h,2h10',1h20' after induction; lane 8, ß-galactosidase, 10 µg; lanes 9-15, soluble proteins 18h,6h10',4h45',4h,3h,2h10',1h20' after induction.

TABLE 1 Expression of hybrid genes after induction by IPTG

Strain	Time (min)	OD(550 nm)	fusion protein (µg/ml culture)
BMH71-18/prANF1			
	80	1.27	0.95
	150	1.80	1.2
BMH71-18/phANF1			
	80	1.35	0.83
	160	1.90	2.92

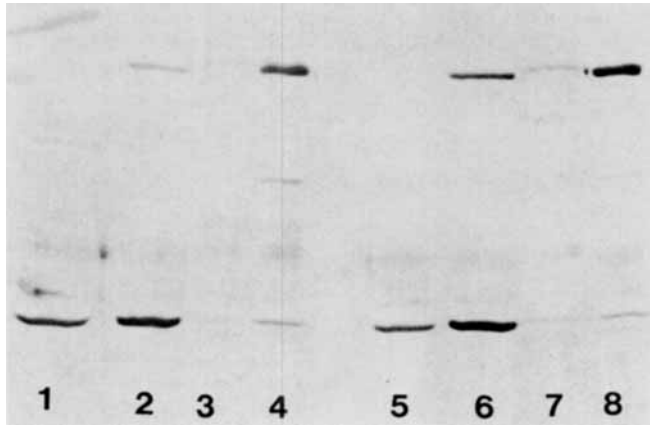

FIGURE 6 Immuno-blot of SDS-PAGE of proteins extracted from BMH71-18/prANF1 and BMH71-18/phANF1
Lane 1, BMH71-18/pUR289; lane 2, BMH71-18/prANF1, soluble proteins 4h after induction; lane 3, BMH71-18,/prANF1, insoluble proteins 18h after induction; lane 4, BMH71-18/prANF1, insoluble proteins 4h after induction; lane 5, BMH71-18/phANF1, soluble proteins 18h after induction; lane 6, BMH71-18/phANF1, soluble proteins 4h after induction; lane 7, BMH71-18/phANF1, insoluble proteins 18h after induction; lane 8, BMH71-18/phANF1, insoluble proteins 4h after induction.

the fractions of insoluble proteins were dissolved in SDS and analysed by SDS-PAGE (Figs. 4,5).

A major protein band, presumably representing the fusion protein ß-galactosidase-rANF and ß-galactosidase-hANF, respectively, increased in amount with time. Quantitative evaluation of SDS-PAGE patterns for the insoluble proteins by densitometry yielded the data detailed in Table 1.

An immuno-blot employing sheep anti-hANF antiserum indicated that the dominant highmolecular weight species contained immuno-reactive ANF (Fig. 6).

Due to the high degree of homology between rANF and hANF a cross-reacticity between rANF and the anti-hANF antiserum was to be expected. The antiserum showed no cross-reaction with ß-galactosidase, although the antiserum obviously contained a relatively high titer of antibodies against other E.coli antigens. This observation was also reported by other authors (3). To suppress this immuno-reactivity, the antiserum was preadsorbed with a crude extract of soluble proteins from E.coli. Despite this treatment, a non ANF-related immuno-reactive polypeptide appeared in the immuno-blot of the fractions of soluble proteins from BMH71-18/pUR289 as well as from BMH71-18/prANF1 and BMH71-18/phANF1. The amount of both fusion proteins went through a maximum, because 18h after induction the SDS-PAGE patterns revealed either the absence (Fig. 4) or significantly reduced amounts of fusion protein (Fig. 5). Densitometric analysis (data not shown) indicated that synthesis of fusion proteins was maximal approximal 4h after induction. It thus appeared that the fusion protein was subject to proteolytic degradation. This finding was paralleled by the results of the immuno-blots.

We successfully cloned the synthetic DNA coding for rANF as well as hANF fused to the lacZ gene as hybrid genes. Induction by IPTG led to a high level of expression of the hybrid genes. As demonstrated, the fusion proteins were immuno-reactive with anti-ANF antiserum, and we expect that the purified fusion proteins should prove useful in experiments as outlined above.

ACKNOWLEDGEMENT

We are indebted to Dr.E.Krauhs for valuable suggestions. We gratefully acknowledge that Dr.F.L.Leidenberger (Institut für Hormon und Fortpflanzungsstörungen, Hamburg) provided us with a Model 381A DNA synthesiser.

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Received March 9, 1987.