REVIEW ARTICLE

The biochemistry and molecular biology of atrial natriuretic factor

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Introduction

It has been known for some 30 years that the cardiocytes of atrial muscle, unlike those of the ventricle, contain electron-dense granules (Kisch, 1956). These granules, termed atrial specific granules by Jamieson & Palade (1964), had no known function and early work on the physiological role of these granules centred around the possibility that they served as a storage site for catecholamines in the heart (Palade, 1961; de Bold & Bencosme, 1973). In 1978, however, de Bold *et al.* suggested that the atrial specific granules had morphological and histochemical properties which were characteristic of granules found in polypeptide-hormone producing cells. In addition, an unbiased morphometric method was developed to assess quantitatively atrial granulation in rats (de Bold, 1978). The development of this methodology allowed the examination of a number of experimental conditions previously reported (Martinez-Palomo & Bencosme, 1966; Bencosme & Berger, 1971; Marie *et al.*, 1976) to affect the degree of atrial granulation and it was demonstrated that, of these various treatments, the induction of sodium deficiency and water deprivation caused significant changes in the number of granules (de Bold, 1979). This apparent relationship between specific atrial granularity and water-electrolyte balance led to the suspicion that the granules were a storage site for a substance involved in the control of water and salt balance. In 1981, de Bold *et al.* (1981) showed that atrial muscle did indeed contain such a factor for, following injection of crude rat atrial muscle extracts, bioassay rats exhibited a marked natriuresis, diuresis and hypotension. This substance was given the name atrial natriuretic factor (ANF). Shortly after, it was demonstrated (de Bold, 1982a) that the specific granules themselves were the likely storage site for ANF. Thus was born the concept that the heart, through the agency of ANF, played an endocrine role in the regulation of extracellular fluid volume and blood pressure.

The studies just described formed the basis of intensive efforts by several laboratories as attempts were made to extract, isolate and identify the nature of ANF. In a remarkable testimony to the techniques of modern biochemistry and to the intense competitiveness engendered by a new and exciting field, this and more was accomplished within 3 years of the initial discovery by de Bold *et al.* (1981) that atrial extracts produced such a marked biological effect following injection into rats. The development of the molecular aspects of the atrial natriuretic factor field over the past 3 years is the subject of this review.

Extraction and purification of ANF from heart tissue

From the very beginning, elucidation of the nature of ANF was dependent on extracting and purifying sufficient amounts for analysis. Almost all of the initial work had been done with rat atria and, given the size of these organs, it was obvious that many thousands would have to be used to eventually produce enough material. Indeed, cognizant of the impending difficulties, de Bold & Salerno (1983) had examined the hearts of several larger species only to find that specific natriuretic activity of atrial extracts was greater in the rat and small rodents than in any other species. This correlated well with a previous observation (Jamieson & Palade, 1964) that the degree of atrial granulation in mammals is approximately inversely proportional to animal size. The first successful attempts at extracting ANF from rat atrial tissue (de Bold 1982a, b) were based on the hypothesis that the atrial specific granules were the sites of storage of ANF. Histologically, it was found that the staining intensity of the granules diminished after fixation in fixatives containing acetic acid and that the staining and histochemical properties indicated the presence of a polypeptide in the granules. Thus, it was logical to use an extractant commonly used for the solution of polypeptides, namely, acetic acid. Initial attempts at purification using the acetic acid extraction technique followed by gel filtration showed that the biological activity of partially purified ANF was completely abolished by proteinase treatment and that, chromatographically, ANF had a multimodal distribution suggesting the presence of several components of various molecular masses (de Bold, 1982b; Thibault *et al.*, 1983a). Indeed, gel-filtration studies indicated that the biological activity was eluted in both high- and low- M_r fractions (Currie *et al.*, 1983; Trippodo *et al.*, 1983) and these studies also substantiated observations of Deth *et al.* (1982) that ANF had smooth muscle relaxant activity.

The first homogeneous preparation of any of these components was obtained by de Bold & Flynn (1983) when purification procedures were modified to incorporate techniques used by Bennett *et al.* (1981) for the purification of pituitary peptides. Strongly acidic extracts were processed through cartridges of octadecylsilylsilica prior to reverse-phase h.p.l.c. By this procedure diuretic and natriuretic activities were recovered in four distinct chromatographic regions, a feature consistently and repeatedly observed in the de Bold laboratory in contrast to that found by other workers (see below). From one of these regions a biologically active peptide was purified to chemical homogeneity. Amino acid analysis showed that this peptide contained two half-cystine residues. No

Abbreviations used: ANF, atrial natriuretic factor; ANP, atrial natriuretic polypeptide; the prefixes h and r refer to the human and rat polypeptides respectively; kb, kilobases or kilobase pairs; bp, base pairs.

Table 1. Amino acid sequence of low- M_r ANF peptides extracted from atrial tissue

The box around the M in α -hANP indicates the only difference in amino acid sequence between low- M_r atrial natriuretic peptides from human and rat. In each case the disulphide bond has been omitted for clarity.

Sequence	Trivial name and reference
S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	Rat atrial peptide [cardionatrin I (Flynn <i>et al.</i> , 1983)]
S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S	Atriopeptin I
S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R	Atriopeptin II
S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	Atriopeptin III
S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S	Des-Ser ¹ -atriopeptin I
C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S	Des-Ser ¹ ,Ser ² -atriopeptin I
S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N	Des-Ser ²¹ -atriopeptin I
R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	Rat ANF (Misono <i>et al.</i> , 1984)
S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	α -hANP (Kangawa & Matsuo, 1984)
L-A-G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	ANF-(1-33)
G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	ANF-(3-33)
R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	ANF-(8-33)
A-L-L-A-G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	β -rANP-(14-28)
L-A-G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	β -rANP-(16-48)
A-G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	β -rANP-(17-48)
G-P-R-S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	β -rANP-(18-48)
S-L-R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	α -rANP [β -rANP-(21-48)]
R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	α -rANP-(4-28)
S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R	α -rANP-(5-28)
R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R	Auriculin A
R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y	Auriculin B

tryptophan was detected either by amino acid analysis or spectrophotometrically. The yield of this peptide (cardionatrin I) was 12–20 nmol/1000 rat atria, indicating the very small amounts of this peptide extractable from heart tissue. [Cardionatrin I, a 49-residue peptide, was the form of ANF peptide initially purified by de Bold & Flynn (1983). It proved to be an *N*-terminally extended form of rat atrial peptide (Flynn *et al.*, 1983) which in subsequent work consistently eluted in the same position as cardionatrin I. The consistent appearance of the 28-residue ANF as the lowest- M_r form led to this being referred to as cardionatrin I. Thus, from this point in the review, in agreement with our latest work (Flynn *et al.*, 1985a), cardionatrin I refers to the 28-residue atrial peptide (Flynn *et al.*, 1983).] Other low- M_r ANFs were purified at about this time (Crammer *et al.*, 1983; Thibault *et al.*, 1983b). In each case there were differences in M_r and amino acid composition. However, it was characteristic of all of them that they had no tryptophan, possessed two half-cystines indicative of the presence of a disulphide bond and were rich in arginine. The differences in amino acid composition were easily explained as amino acid sequences of these different ANFs became available. They were all extended or truncated versions of a peptide which contained, as a basic core, a 17-residue, disulphide bonded loop (Table 1).

Amino acid sequence studies

The amino acid sequences of low- M_r ANFs from rat heart were published virtually simultaneously by a

number of laboratories (Flynn *et al.*, 1983; Currie *et al.*, 1984a; Kangawa *et al.*, 1984a; Seidah *et al.*, 1984) and shortly after sequences of higher- M_r forms appeared (Thibault *et al.*, 1984a). At about the same time Kangawa & Matsuo (1984) published the sequence of an ANF peptide from human heart which they called α -human atrial natriuretic polypeptide (α -hANP). There was a striking homology between this 28-residue peptide and cardionatrin I (Flynn *et al.*, 1983) in that the only difference was a methionine in place of an isoleucine at position 12. Such evolutionary conservation of primary structure indicates retention of a vital and indispensable function (Wilson *et al.*, 1977) and the lack of homology of the ANF sequences with any other protein of known structure served to emphasize the discovery of a truly unique and novel peptide. Indeed, the biological action of ANF peptides at sites distant from the heart strongly suggested the discovery of a new endocrine system. Further evidence that the heart acted in an endocrine capacity, however, required demonstration of the presence of ANF peptides in the blood.

Initially, mainly due to the lack of a radioimmunoassay, detection of atrial natriuretic peptides in the blood was not feasible and attention was given to the relationship between the various low- M_r ANFs (Table 1) and between these and the higher- M_r forms. This multiplicity of different ANF peptides also gave rise to the notion of a family of peptides (Geller *et al.*, 1984a). However, the large variety of different ANFs, extracted from atrial tissue by different laboratories, all with the same 17-residue disulphide-bonded core, suggested that at least

some had been produced artifactually during the extraction and purification process. Indeed, Kangawa *et al.* (1984b) showed that α -rANP-(5-27), a peptide identical with atriopeptin III (Geller *et al.*, 1984a) was isolated when heat treatment was carried out after extraction and that the use of fresh tissue and heat treatment prior to homogenization was essential for preventing proteolysis during extraction. Despite this precaution these authors nevertheless extracted and purified seven low- M_r ANF peptides from rat atrial tissue (see Table 1). In contrast with this, and with the results of other laboratories shown in Table 1, we have consistently observed only four peptides in extracts of rat atrial muscle (Flynn *et al.*, 1985a). Only one of these, cardionatrin I (Table 1) can be classified as a low- M_r ANF. From a comparison of our procedure (de Bold & Flynn, 1983; Flynn *et al.*, 1985a) with that from other laboratories it is difficult to pinpoint what particular step, if any, caused artifactual proteolysis. The same basic procedure was used by all laboratories: homogenization of freshly excised tissue, acetic acid extraction, initial processing by gel-permeation chromatography and final purification by several h.p.l.c. applications. Some laboratories used no proteinase inhibitors and avoided a heating or boiling step (de Bold & Flynn, 1983). Thibault *et al.* (1983b) included proteinase inhibitors but avoided heating. Other workers (e.g. Kangawa *et al.*, 1984a) boiled diced atrial tissue in 1 M-acetic acid prior to processing, whereas Currie *et al.* (1984a) boiled the supernatants obtained following centrifugation of rat atria homogenized in solutions of proteinase inhibitors. Thus, it is not clear which of the large variety of low- M_r ANFs comprise the actual forms present in atrial tissue. The absolute consistency of the procedure of de Bold & Flynn (1983) in extracting a total of only four atrial natriuretic peptides from atrial tissue which included but a single low- M_r form (cardionatrin I; Flynn *et al.*, 1985a) suggests that these peptides are the ones present in atrial tissue.

It was clear from the sequencing studies that all of the extracted ANFs ended in a tyrosine residue (Table 1) and that no ANF had been extracted which extended beyond this residue. This observation, coupled with the fact that several peptides with varying *N*-terminal extensions had been sequenced (Thibault *et al.*, 1984a; Napier *et al.*, 1984; Seidah *et al.*, 1984; Kangawa *et al.*, 1984a, b), strongly supported the idea of precursor-product relationship for low- M_r ANFs. It had clearly been implied from the work of de Bold (1982b), de Bold & Flynn (1983), Currie *et al.* (1983) and Trippodo *et al.* (1984) that higher- M_r biologically active forms of ANF existed. Indeed, it had been shown prior to sequencing studies that higher- M_r ANF could be converted into lower- M_r forms by gentle proteolysis (Currie *et al.*, 1984b), or by mixing higher- M_r ANF with crude atrial extract (Trippodo *et al.*, 1984).

Before determination of the total amino acid sequence of the precursor had been completed, several higher- M_r ANFs containing the sequence of low- M_r ANF at their C-termini had been sequenced (Table 2). The longest of these had 111 residues and was called atriopeptigen (Geller *et al.*, 1984b). The first 15 amino acids of this peptide and the first 10 of the 106-residue peptide termed pronatriodilatin by Lazure *et al.* (1984) showed a high degree of homology with the C-terminal portion of the first 30 residues of a 7500-Da peptide extracted from pig

Table 2. Amino acid sequence of high- and intermediate- M_r atrial natriuretic peptides extracted from atrial tissue

1	2
K-N-L-L-D-H-L-E-E-K-M-P-V-E-D-	
	E-V-M-P-P-Q-A-L-S-E-Q-T-D-E-A-
	3
G-A-A-L-S-S-L-S-E-V-P-P-W-T-G-	
	E-V-N-P-S-Q-R-D-G-G-A-L-G-R-G-
	P-W-D-P-S-D-R-S-A-L-L-K-S-K-L-
	R-A-L-L-A-G-P-R ₁ S-L-R-R-S-S-C-
	F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-
	C-N-S-F-R-Y

¹The total sequence given is that of atriopeptogen (Geller *et al.*, 1984b). ²The sequence beginning H-L-E... is the *N*-terminal end of a 106-residue peptide termed atrial pronatriodilatin sequenced by Lazure *et al.* (1984). ³The sequence beginning E-V-P... is the *N*-terminal end of rat ANF-H1 sequenced by Thibault *et al.* (1984a). The broken line encloses the sequence of biologically active rat atrial peptide [cardionatrin-(1-28)] (Flynn *et al.*, 1983).

heart (Forssmann *et al.*, 1983). This peptide, called cardiodilatin, was said to have only smooth muscle relaxant activity and to be devoid of diuretic and natriuretic activity. A subsequent paper by Forssmann *et al.* (1984) casts doubt on this finding. Cardiodilatin also exhibited no homology with any other known protein.

The novel nature of both pig cardiodilatin and rat and human ANF sequences together with the almost perfect match of 15 amino acids strongly suggested that both cardiodilatin and ANF were part of the same precursor molecule. By aligning the homologous sequences in cardiodilatin and atriopeptogen and/or pronatriodilatin, it was clear that the precursor molecule was at least 126 amino acids long. As it turned out this observation was remarkably accurate, as precisely this number of amino acids was found to comprise proatrialnatriuretic factor when the cDNA for the rat and human natriuretic factor was cloned and sequenced.

cDNA Cloning and sequencing

The nature of the ANF precursor was succinctly revealed by DNA sequencing of cDNA clones (Fig. 1). In the rat and mouse the primary translation product is 152 amino acids long, while the human counterpart is one residue shorter. The most commonly used strategy for the isolation of ANF cDNA clones proved to be the screening of atrial cDNA libraries with sets of end-labelled, mixed-based oligonucleotide probes. The protein sequences available at this time for the design of probes were those of the shorter ANF peptides from the C-terminal end of the precursors. To select rat ANF cDNA clones, Yamanaka *et al.* (1984) and Maki *et al.* (1984b) used sets of tetradecanucleotides corresponding

Fig. 1. Sequence of rat ANF cDNA

The DNA sequence is a composite of a cDNA sequence (Flynn *et al.*, 1985a) extended to the cap site as determined from the sequence of a genomic clone (Argentin *et al.*, 1985). The rat ANF precursor sequence is written below. The *N*-terminal sequence of cardionatrin IV is indicated by horizontal arrows beginning at residue 25 and the entire cardionatrin I sequence by horizontal arrows beginning at residue 123. Two putative polyadenylation signals are underlined and the two vertical arrows at the end of the sequence indicate sites of polyadenylation on the mRNA.

to the pentapeptide sequences between residues 129 and 133. To reduce the number of false positives emerging from the screening procedure the clones were challenged with a second set of tetradecamers matching the protein sequences between residues 134 and 138 (Yamanaka *et al.*, 1984) or between residues 145 and 149 (Maki *et al.*, 1984b). A similar double-barrelled approach to the screening was used by Seidman *et al.* (1984a). In general, clones which reacted positively to both sets of probes proved to be ANF cDNA clones. The redundancy of the codons corresponding to these regions of rat ANF typically required the synthesis of 96 different tetradecamers per set. However, in human ANF the presence of methionine in place of isoleucine at position 135 not only simplified the choice of a region to which to make probes but also reduced the complexity of the probe mixture. Both Oikawa *et al.* (1984) and Nakayama *et al.* (1984) were able to use just 36 tetradecamers corresponding to residues 135–139 of the human ANF precursor to isolate human ANF cDNA clones. A comparable reduction in the complexity of oligonucleotide probes used for the isolation of rat ANF cDNA (Zivin *et al.*, 1984) was achieved by making tetradecamers to a pentapeptide sequence (residues 82–86) which was revealed in the sequence of one of the longer ANF-containing peptides.

A different approach to the isolation of rat ANF cDNA

clones, taken by Kennedy *et al.* (1984), was formulated around the assumption that ANF would be one of the few sequences expressed in the atria but not in the ventricles. Duplicate clones from an atrial cDNA library were screened by differential colony hybridization using oligo(dT)₁₀-primed atrial cDNAs and ventricular cDNAs as probes. Two out of 800 clones were identified as being atrial-specific. DNA sequence analysis showed them to be truncated ANF cDNA clones which likely had suffered from over-digestion by S₁ nuclease during the cloning procedure. These sequences were subsequently used to select longer rat ANF clones from another cDNA library (Flynn *et al.*, 1985a).

The similarity in nucleotide sequence between rat and human ANF mRNAs can result in hybridization between their cDNA clones. This property enabled Zivin *et al.* (1984) to isolate human ANF cDNA clones with a rat ANF cDNA probe, and Kangawa *et al.* (1984c) to do the converse experiment.

The structure of the ANF precursor

The primary structure of the ANF precursor (Fig. 2) was deduced from the DNA sequence of ANF cDNA clones by locating the reading frame containing the C-terminal ANF peptides and following its *N*-terminal extension to the methionine at residue 1. The identification of this residue as the initiating methionine is supported

Fig. 2. Restriction maps of genomic clones containing the rat ANF gene

The three overlapping clones (J1–3) were isolated from 4×10^5 recombinant phage as described by Argentin *et al.* (1985). The rat DNA inserts are represented by the horizontal line between the long (L.A.) and short (S.A.) arms of the λ Charon 30 vector. The underlining shows the location of the ANF gene. The restriction sites mapped were *Hind*III (H), *Eco*RI (E), *Bam*HI (B) and *Kpn*I (K). The asterisks represent *Bam*HI sites generated during the cloning.

by the resemblance of the next 20+ residues to a classic signal polypeptide sequence (Perlman & Halverson, 1983). The human ANF precursor has a hydrophobic core from residues 11 to 17 and the rat precursor one from residues 10 to 18. The predicted cleavage points for the removal of the signal sequence, according to the observations of Perlman & Halverson (1983), would be after the alanine at residue 25 in the human ANF precursor and after the alanine at residue 24 in the rat equivalent. Confirmation of these predictions came from the *N*-terminal sequence analysis of the largest ANF precursors to be isolated from atrial tissue. In human atria this molecule is the 126-residue γ -hANP which begins at residue 26 with the sequence NH_2 -Asn-Pro-Met-Tyr... (Oikawa *et al.*, 1984). In rat atria the equivalent precursor is also 126 residues long and starts at residue 25 with the analogous sequence NH_2 -Asn-Pro-Val-Tyr... It has been referred to as γ -rANP (Kangawa *et al.*, 1984c) and cardionatrin IV (Flynn *et al.*, 1985a). The *N*-terminal sequences of these large ANF precursors are homologous to those of the cardiodilatin peptides extracted from pig atria (Forssmann *et al.*, 1983; 1984). Based on the DNA sequence one would predict that the rat ANF precursor might be larger than its human equivalent by the two arginine residues encoded at the very C-terminus following tyrosine 126. In fact, none of the rat ANF peptides or precursors have been isolated with these two basic residues in place, and their removal from the primary translation product must be extremely rapid. The DNA sequence difference that leads to this C-terminal extension in the rat is discussed later.

The presence of a signal sequence on the ANF precursor is to be expected in view of the accumulation of ANF peptides in secretory granules and their hormonal nature. The remainder of the precursor could be considered as a proprotein because it encompasses the smaller ANF peptides. However, this begs the question of the true nature of the hormone. Is it the 126-residue protein or one of the smaller ANF peptides? In terms of abundance both the 126-residue protein (γ -hANP) and the 28-residue ANF peptide (α -hANP) are recovered from human atria in good yield (Kangawa *et al.*, 1985). In the rat, the protein is far more abundant than the peptide, both in atrial extracts (Kangawa *et al.*, 1984c; de Bold *et al.*, 1985) and atrial-specific granules (Flynn *et al.*, 1985b). In rat plasma two main peaks of

immunoreactive ANF are found after reverse-phase h.p.l.c. (Davies *et al.*, 1985). These peaks have chromatographic mobilities which are identical with those of cardionatrin I and IV respectively. These findings suggest that ANF is stored mainly as cardionatrin IV in atrial muscle. Thus the protein may be processed prior to release from the heart to yield cardionatrin I or it may be released as such and processed in the blood to a low-*M_r* form.

Isolation of the gene for atrial natriuretic factor

Aside from revealing the structure of the ANF precursor the ANF cDNA sequences have provided access to the gene. When Southern blots of *Bam*HI-restricted and *Eco*RI-restricted rat, mouse and human genomic DNAs were probed with labelled ANF cDNA, a single band of intense hybridization was seen in each species (Seidman *et al.* 1984a). Since the authors performed the hybridization under conditions where sequences of less than 70% homology should not hybridize, they concluded that the gene sequence was well conserved between these species, and that it possibly existed as a single copy sequence. Two very faint bands of hybridization were seen at other positions on the blot. A more extensive analysis of human genomic DNA (Okikawa *et al.*, 1984) with two other enzymes (*Hind*III and *Bcl*I) and of rat genomic DNA (Argentin *et al.*, 1985) involving four other restriction enzymes (*Hind*III, *Kpn*I, *Sst*I and *Xba*I), supported the single-copy status of the rat ANF gene, although again some very faint traces of hybridization were seen to other restriction fragments. The rat ANF gene was contained within a 4.2 kb *Eco*RI fragment and a 4.1 kb *Hind*III fragment, and the human ANF gene within a 3.3 kb *Bam*HI fragment.

Making use of the ANF gene homologies between species, the rat ANF cDNA was used to isolate ANF genomic clones from λ Charon 4A libraries of human DNA (Nemer *et al.*, 1984; Greenberg *et al.*, 1984; Maki *et al.*, 1984a; Seidman *et al.*, 1984b) and from a plasmid library of size-selected *Bam*HI-digested mouse DNA (Seidman *et al.*, 1984b). Subsequently the rat ANF gene was isolated from a λ Charon 30 rat DNA library by the same technique (Argentin *et al.*, 1985). The ANF gene was recovered in λ library screenings at a frequency of 2 in 5×10^5 clones (Maki *et al.*, 1984a), 5 in 5×10^5 clones (Seidman *et al.*, 1984b) and 3 in 4×10^5 clones (Argentin

et al., 1985) consistent with it being a single copy sequence.

Intron-exon structure

The human (Greenberg *et al.*, 1984), rat (Argentin *et al.*, 1985) and mouse (Seidman *et al.*, 1984b) ANF genes have been sequenced in their entirety together with substantial amounts of 5' and 3' flanking DNA. A comparison of these sequences with those of their respective cDNAs shows that the gene is interrupted twice by introns. The first and smaller intron, which is 122 bp, 104 bp and 103 bp long in the human, rat, and mouse genes respectively, interrupts the gene right after the DNA coding for the 16th residue of pro-ANF. The second and larger intron interrupts the gene just before the DNA coding for the C-terminal tyrosine. Thus in the human gene the third exon codes for a single residue followed by the 3' untranslated region of the mRNA, whereas in the rat gene two additional arginine residues are encoded. A comparison of the rat and human ANF gene sequences in this region shows a single base change (C $\leftrightarrow$ T) immediately after the tyrosine codon. In the human gene the following codon is TGA, a termination codon, whereas in the rat gene it is an arginine codon, CGA (Fig. 1). Immediately after a second arginine codon is an in-frame stop codon, TAA. This single base difference is very close to the splice site for the second intron. After splicing in the rat a *KpnI* site is formed. In the human ANF gene sequence the single base change coincides with the 3' end of the *KpnI* recognition site such that this enzyme cannot cut in the equivalent position in this gene. However, Nemer *et al.* (1984) report the cloning of a variant human ANF cDNA in which the base in this position is C as in the rat, rather than T. This change is confirmed by the presence of a *KpnI* cleavage site in the region. Since their Southern blot experiments also suggested the presence of only one ANF gene the difference observed by Nemer *et al.* (1984) in the two sequences was thought to be allelic.

The second intron is 391 bp long in the rat (Argentin *et al.*, 1985) but considerably larger (1093 bp) in the human ANF gene (Greenberg *et al.*, 1984), largely because it contains two tandem *Alu* repeat sequences (Deininger *et al.*, 1981) which are 76% homologous. Sequence homology between the rat and human second introns is confined to the ends and there are no equivalents to the *Alu* repeat sequences within the rat gene. Seidman *et al.* (1984b) and Greenberg *et al.* (1984) report a potential glucocorticoid receptor binding site in the second intron of the human gene, but the significance of this observation is unclear. Greenberg *et al.* (1984) have also commented on the positioning of the introns in respect to the hypothesis of Gilbert (1978) that functional or structural domains are delineated by exons. The second intron separates only the C-terminal tyrosine from the remainder of the hormone. Although deletion of this residue does affect slightly the binding of ANF to its receptor (Garcia *et al.*, 1985) it is not entirely clear whether or not the C-terminal residue is absolutely essential for biological activity (Wakitani *et al.*, 1985). The site of the first intron, coming 16 residues after the signal peptide splice site, is well removed from the C-terminal ANF peptide and does not correlate with any obvious discontinuity in the structure of the precursor.

Gene structure and homologies

The structural features of the ANF gene are quite conventional. About 110 bp upstream of the initiation codon is a typical TATA box. Although potential CAAT boxes do occur further upstream they are not very close matches to this consensus sequence (Greenberg *et al.*, 1984). Downstream of the TATA box, approximately 95 bp before the initiation codon, is the probable start point for transcription. This site has been established by extension of a synthetic primer on human atrial mRNA (Nemer *et al.*, 1984). The cap site in the rat gene was similarly located by primer extension (Zivin *et al.*, 1984) in relation to the genomic sequence (Argentin *et al.*, 1985). The intron-exon junction sequences are typical of those found in other eukaryotic genes (Breathnach & Chambon, 1981). A polyadenylation signal AATAAA is located in the human gene approximately 20 bp upstream of the poly(A) attachment site, whereas the rat gene contains two such signals in the same vicinity. It is not clear if the duplication of this signal in the rat can explain the heterogeneity observed in the poly(A) attachment sites (Kennedy *et al.*, 1984; Flynn *et al.*, 1985a).

One interesting structural feature appears near the rat gene in the 3' flanking DNA. A little over 100 bp downstream from the polyadenylation site is a long alternating purine-pyrimidine sequence (GT)₆GATG(GT)₂₇ (Argentin *et al.*, 1985). Sequences of this nature have been associated with Z-DNA formation (Nordheim & Rich, 1983; Wells *et al.*, 1982) and recombination (Slighton *et al.*, 1980; Stoeckert *et al.*, 1984). A run of eight alternating GTs has been observed in the equivalent region of the mouse gene (Seidman *et al.*, 1984b), but based on hybridization to a (GT)₁₂ probe there is no evidence for a similar tract flanking the 3' side of the human ANF gene (Argentin *et al.*, 1985).

Approximately 14 kb of genomic DNA encompassing the human ANF gene have been restriction mapped (Nemer *et al.*, 1984). In the rat a set of three overlapping genomic clones have contributed to the mapping of 27 kb of the rat genomic around the gene (Fig. 2). At this gross level of structural analysis there is no obvious similarity in the positioning of restriction sites between the two species. Both within and around the gene homologies between species can now be reported from direct sequence comparisons. In the amino acid sequences there are 17 changes between the rat and human ANF propeptides (Greenberg *et al.*, 1984; Kangawa *et al.*, 1984c). That is a homology level of 85% identity. A similar value obtains for the nucleotide sequence. Within the propeptide the N-terminal portion, and the C-terminal portion encompassing the 28-residue ANF peptide, are the most highly conserved regions. This degree of homology explains the success of the heterologous probes used in the genomic Southern blots and library screenings. The signal sequence is not so well conserved. There is only 52% homology in the amino acid sequences and 70% homology at the nucleotide level.

In comparing the other gene regions (Argentin *et al.*, 1985), the intervening sequences predictably are poorly conserved but the 5' flanking sequences show 85% homology to at least 200 bp upstream of the cap site. This may reflect the need to preserve regulatory sequences in this region.

Direct comparisons of the mouse and human ANF nucleotide sequences and the mouse and rat amino acid

sequences have been reported by Seidman *et al.* (1984b). In comparing the two rodent sequences the same pattern of homology emerges as for the rat and human comparison. There are a total of four amino acid substitutions within the ANF proprotein, none of which occur in the *N*-terminal and *C*-terminal portions, and there are two substitutions within the signal sequence. The first 30 amino acid residues of pig ANF proprotein can be compared from the published sequence of cardiodilatin (Forssmann *et al.*, 1983, 1984). There are four substitutions in this region in comparison with the human sequence and five in comparison with the rodent sequences.

Future comparative studies of this nature are likely to focus on the sequence upstream of the gene in a search for regulatory elements.

Rat ANF mRNA: its expression and regulation

The mature ANF mRNA from rat is a little over 800 nucleotides long not including the poly(A) tail (Fig. 1). On Northern blots of atrial RNA the mRNA hybridizes as a band at 850–900 nucleotides in length (Yamanaka *et al.*, 1984; Seidman *et al.*, 1984a). Based on these and other Northern blots (Kennedy *et al.*, 1984; Zivin *et al.*, 1984) the ANF mRNA is quite abundant in the atria but not detectable by this technique in RNA from the ventricles. A very rough estimate of its abundance can be made from scoring the percentage of cDNA clones in a random atrial cDNA library that hybridize to a defined ANF cDNA probe. Yamanaka *et al.* (1984) found 0.5% of their atrial cDNA clones were ANF sequences, while the value obtained by Maki *et al.* (1984b) was closer to 1%, and the value indicated by Flynn *et al.* (1985a) was 2%. Seidman *et al.* (1984b) report the abundance of ANF mRNA as being 1–3% of atrial mRNA, and Nakayama *et al.* (1984) as 0.5–1%.

These approximate values for ANF mRNA levels pertain to rats that are presumably fed *ad libitum*. In view of the role of ANF in cardiovascular homeostasis and the pronounced effect that perturbations in the water–electrolyte balance have on the presumptive ANF storage granules, it is of interest to see what effect such perturbations have on the levels of ANF mRNA in the atria. Nakayama *et al.* (1984) have examined the effect of water deprivation on ANF mRNA levels in rat atria. After 2 and 4 days without water ANF mRNA levels were about two and three times lower than that in control rats respectively. In the 4 day water-deprived rats the yield of RNA from the atria was also decreased to two-thirds of that obtained from control rats. Therefore, the overall decrease in ANF mRNA by day 4 was enhanced. We have recently done a similar study in which, to circumvent fluctuations in RNA yields, the ANF mRNA levels have been related directly to the levels of an internal standard RNA, actin mRNA (Davies, P. L., Hegadorn, C. A., Tadross, O. & de Bold, A. J., unpublished work). After 5 days of water deprivation a 4-fold decrease in ANF mRNA levels was also observed by these criteria. It is not known at this time whether this reflects a decrease in the rate of transcription of the gene or a change in mRNA metabolism. Additional investigations are clearly warranted in order to understand the regulation of ANF production, both in normal and in pathological conditions.

Perspectives and prospects

Ever since the observation was made (de Bold *et al.*, 1981) that the injection of crushed atrial extracts into rats caused such a marked physiological response, the aim of researchers has been directed towards defining the role of ANF in the regulation of sodium and water balance and blood pressure. The clinical implications of ANF are also receiving considerable attention (for a recent commentary on this see Mills, 1984) since it has long been suspected that there is a connection between essential hypertension and the kidney's ability to excrete sodium (de Wardener & MacGregor, 1983). Further, the induced diuresis following distension of the atria and the response of the kidney to increases in blood volume (for recent reviews see Cantin & Genest, 1985; Napier & Blaine, 1984) clearly imply a role for ANF in such diseases as congestive heart failure. However, it is evident that much of the current and future physiological and pharmacological research on ANF could not proceed without knowledge of the biochemical information on ANF and its precursor uncovered over the past few years. This is particularly true for the elucidation of the primary structure of low- M_r ANF with the basic 17-residue disulphide core, which, to us, represents the pivotal point in research on ANF. The very small amounts of ANF extractable from heart tissue practically precluded any full-scale physiological investigations, but once the sequence was known, chemical synthesis of biologically active ANF peptides became feasible. Chemical synthesis of ANF, first accomplished by Ruth Nutt and her colleagues at Merck, Sharpe & Dohme (Seidah *et al.*, 1984), in fact became the key element for any proposed research on ANF and its effects. Knowledge of the sequence of ANF is also essential for the development of chemical analogues by drug companies.

The most immediate use, however, for synthetic ANF peptides was the development of a radioimmunoassay, since amounts of extractable ANF had never been sufficient for this purpose. Using polyclonal antibodies raised in rabbits to a variety of low- M_r ANF peptides, several groups detected and measured ANF in atria and plasma (Gutkowska *et al.*, 1984a, b, c; Tanaka *et al.*, 1984; Nakao *et al.*, 1984) and in several parts of the brain (Mori *et al.*, 1985). This latter research, together with that of the de Bold group (Davies *et al.*, 1985), is significant in that it shows that the predominant form in plasma is low- M_r ANF whereas, in contrast, the major immunoreactive form in atria appears to be the 13 kDa form (pro-ANF factor minus the two *C*-terminal arginine residues).

Synthetic ANF peptides consisting of the 17-residue disulphide-bonded core together with various numbers of residues at *N*- and *C*-termini have proved useful in establishing the minimum structure necessary for biological activity. Reduction of the disulphide loop totally abolishes biological activity (Currie *et al.*, 1984b) and it is apparent that almost the complete *C*-terminal sequence is required for both smooth-muscle relaxing activity and for natriuresis (Thibault *et al.*, 1984b; Garcia *et al.*, 1985; Currie *et al.*, 1984b). It is not yet clear from results presented so far that the *C*-terminal tyrosine residue is absolutely essential. Truncation of *N*-terminal residues from ANF peptides has little effect on biological activity.

The availability of synthetic ANF peptides has also

enabled detection of specific ANF receptors in the kidney and blood vessels and allowed examination of their binding characteristics (Napier *et al.*, 1984; Hirata *et al.*, 1984; Schenk *et al.*, 1985). In addition, Yip *et al.* (1985) have shown recently, by using photoaffinity-labelled ^{125}I -labelled rat cardionatriin I, that the rat kidney receptor is a glycoprotein of M_r 140000. Isolation of the receptor will enable structure-function aspects of ANF and its receptor to be examined in much greater detail.

Synthetic ANF peptide availability has also stimulated a great deal of activity in metabolic aspects of ANF. Of particular interest is the finding that ANF probably exerts its effects through a cyclic GMP-dependent mechanism (Winquist *et al.*, 1984).

The scope of this article does not allow a thorough survey of all of the literature concerning ANF. Suffice it to say that since the amino acid sequence of low- M_r ANF was published and synthetic ANF peptides became available, well over 100 papers have been published in relation to ANF. More and more attention is now being paid to the mechanism of action of ANF and related peptides at the physiological and pharmacological levels, and this undoubtedly will be the main focus of research in this area for some time. However, there will be more biochemistry and molecular biology to come, particularly in relation to the biosynthesis of ANF and its precursor, to the processing of the pro-ANF and its subsequent release and to the control of synthesis and release at the gene level. In addition, much awaits to be done in the area of the structure of the receptor and on any processes of internalization of receptor and subsequent recycling. If progress in the ANF field continues at its current pace there is every expectation that a very detailed picture of the physiology, pharmacology and biochemistry of ANF will emerge in the near future.

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REFERENCES

- Argentin S., Nemer M., Drouin J., Scott, G. K., Kennedy, B. P. & Davies, P. L. (1985) *J. Biol. Chem.* **260**, 4568–4571
- Atlas, S. A., Kleinert, H. D., Camargo, M. J., Januszewicz, A., Sealey, J. E., Laragh, J. H., Schilling, J. W., Lewicki, J. A., Johnson, L. K. & Maack T. (1984) *Nature (London)* **309**, 717–719
- Bencosme, S. A. & Berger, J. M. (1971) in *Methods and Achievements in Experimental Pathology* (Bajusz E. & Jasmin G., eds.), vol. 5, pp. 173–213, Karger, Basel
- Bennett, H. P. J., Browne, C. A. & Solomon S. (1981) *Biochemistry* **20**, 4530–4538
- Breathnach R. & Chambon P. (1981) *Annu. Rev. Biochem.* **50**, 349–383
- Cantin M. & Genest J. (1985) *Endocr. Rev.* **6**, 107–127
- Crammer, R. T., Fukuni H., Inagami T. & Misono, K. S. (1983) *Biochem. Biophys. Res. Commun.* **116**, 696–703
- Currie, M. G., Geller, D. M., Cole, B. R., Boylan, J. G., YuSheng W., Holmberg, S. W. & Needleman P. (1983) *Science* **221**, 72–73
- Currie, M. G., Geller, D. M., Cole, B. R., Siegel, N. R., Fok, K. F., Adams, S. P., Eubanks, S. R., Gallupi, G. R. & Needleman, P. (1984a) *Science* **223**, 67–69
- Currie, M. G., Geller, D. M., Cole, B. R. & Needleman P. (1984b) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1230–1233
- Davies, P. L., de Bold, A. J., de Bold, M. L., Flynn, T. G. & Kennedy, B. P. (1985) *Proc. Can. Fed. Biol. Soc.* **28**, 164
- de Bold, A. J. (1978) *J. Mol. Cell. Cardiol.* **10**, 717–724
- de Bold, A. J. (1979) *Proc. Soc. Exp. Biol. Med.* **161**, 508–511
- de Bold, A. J. (1982a) *Can. J. Physiol. Pharmacol.* **60**, 324–330
- de Bold, A. J. (1982b) *Proc. Soc. Exp. Biol. Med.* **170**, 133–138
- de Bold, A. J. & Bencosme, S. A. (1973) *Cardiovasc. Res.* **7**, 351–363
- de Bold, A. J. & Flynn, T. G. (1983) *Life Sci.* **33**, 297–302
- de Bold, A. J. & Salerno T. (1983) *Can. J. Physiol. Pharmacol.* **61**, 127–130.
- de Bold, A. J., Raymond, J. J. & Bencosme, S. A. (1978) *J. Histochem. Cytochem.* **26**, 1094–1102
- de Bold, A. J., Borenstein, H. B., Veress, A. T. & Sonnenberg H. (1981) *Life Sci.* **28**, 89–94
- de Bold, A. J., Davies, P. L., de Bold M., Flynn, T. G., Kennedy, B. P., Marsden, J. J., Sarda, I. R. & Tadross, O. (1985) *Proc. Naito Found. Symp. Nat. Comps. (Japan)*, in the press
- Deininger, P. L., Jolly, D. J., Rubin, C. M., Friedmann T. & Schmid, C. W. (1981) *J. Mol. Biol.* **151**, 17–33
- Deth, R. C., Hong K., Fukagawa S., Rocco R., Smart, J. L., Lynch, C. J. & Wawad R. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 983a
- de Wardener, H. E. & MacGregor, G. A. (1983) *Medicine* **62**, 310–326
- Flynn, T. G., de Bold, M. L. & de Bold, A. J. (1983) *Biochem. Biophys. Res. Commun.* **117**, 859–865
- Flynn, T. G., Davies, P. L., Kennedy, B. P., de Bold, M. L. & de Bold, A. J. (1985a) *Science* **228**, 323–325
- Flynn, T. G., de Bold, A. J., de Bold, M. L., Davies, P. L. & Kennedy, B. P. (1985b) *Biochem. Soc. Trans.*, **13**, 1141
- Forssmann, W. G., Hock D., Lottspeich F., Henschen, A., Kreye V., Christmann M., Reinecke M., Metz J., Carlquist M. & Mutt V. (1983) *Anat. Embryol.* **168**, 307–313
- Forssmann, W. G., Birr C., Carlquist M., Christmann M., Finke R., Henschen A., Hock D., Kirchheim H., Kreye V., Lottspeich F., Metz J., Mutt V. & Reinecke M. (1984) *Cell Tissue Res.* **238**, 425–430
- Garcia R., Thibault G., Seidah, N. G., Lazure C., Cantin M., Genest J. & Chretien M. (1985) *Biochem. Biophys. Res. Commun.* **126**, 178–184
- Geller, D. M., Currie, M. G., Wakitani K., Cole, B. R., Adams, S. P., Fok, K. F., Siegel, N. R., Eubanks, S. R., Gallupi, G. R. & Needleman P. (1984a) *Biochem. Biophys. Res. Commun.* **120**, 333–338
- Geller, D. M., Currie, M. S., Siegel, N. R., Fok, K. F., Adams, S. P. & Needleman P. (1984b) *Biochem. Biophys. Res. Commun.* **121**, 802–807
- Gilbert W. (1978) *Nature (London)* **271**, 501–502
- Greenberg, B. D., Bencen, G. H., Seilhamer, J. J., Lewicki, J. A. & Fiddes, J. C. (1984) *Nature (London)* **312**, 656–658
- Gutkowska J., Thibault G., Millne, R. W., Januszewicz P., Schiller P., Cantin M. & Genest J. (1984a) *Proc. Soc. Exp. Biol. Med.* **176**, 105–108
- Gutkowska J., Thibault G., Januszewicz P., Cantin M. & Genest J. (1984b) *Biochem. Biophys. Res. Commun.* **122**, 593–601
- Gutkowska J., Horky K., Thibault G., Januszewicz P., Cantin M. & Genest J. (1984c) *Biochem. Biophys. Res. Commun.* **125**, 315–323
- Hirata Y., Tomita M., Yoshimi H. & Masao I. (1984) *Biochem. Biophys. Res. Commun.* **125**, 562–568
- Jamieson, J. D. & Palade, G. E. (1964) *J. Cell Biol.* **23**, 151–172
- Kangawa K. & Matsuo H. (1984) *Biochem. Biophys. Res. Commun.* **118**, 131–139
- Kangawa K., Fukuda A., Minamino N. & Matsuo H. (1984a) *Biochem. Biophys. Res. Commun.* **119**, 933–940
- Kangawa K., Fukuda A., Kubota I., Hayashi Y. & Matsuo, H. (1984b) *Biochem. Biophys. Res. Commun.* **121**, 585–591
- Kangawa K., Tawaragi Y., Oikawa S., Mizuno A., Sakuragawa Y., Nakazato H., Fukuda A., Minamino N. & Matsuo H. (1984c) *Nature (London)* **312**, 152–155
- Kangawa K., Fukuda A. & Matsuo H. (1985) *Nature (London)* **313**, 397–400

- Kennedy, B. P., Marsden, J. J., Flynn, T. G., de Bold, A. J. & Davies, P. L. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1076–1082
- Kisch B. (1956) *Exp. Med. Surg.* **14**, 99–112
- Lazure C., Seidah, N. G., Chretien M., Thibault G., Garcia, R., Cantin M. & Genest J. (1984) *FEBS Lett.* **172**, 80–86
- Maki M., Parmentier M. & Inagami T. (1984a) *Biochem. Biophys. Res. Commun.* **125**, 797–802
- Maki M., Takayanagi R., Misono, K. S., Pandey, K. N., Tibbetts C. & Inagami T. (1984b) *Nature (London)* **309**, 722–724
- Marie, J. R., Guillemot H. & Hyatt, P. Y. (1976) *Pathol. Biol.* **24**, 549
- Martinez-Palono A. & Bencosme, S. A. (1966) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25**, 476a
- Mills, I. H. (1984) *Br. Med. J.* **289**, 210–211
- Misono, K. S., Fukumi H., Grammer, R. T. & Inagami T., (1984) *Biochem. Biophys. Res. Commun.* **119**, 524–529
- Morii N., Nakao K., Sugawara A., Sakamoto M., Suda, M., Shimokura M., Kiso Y., Kihara M., Yamori Y. & Imura H. (1985) *Biochem. Biophys. Res. Commun.* **127**, 413–419
- Nakao K., Sugarana A., Morii N., Sakamoto M., Suda, M., Soneda J., Ban T., Kihara M., Yamori Y., Shimokura M., Kiso Y. & Imura H. (1984) *Biochem. Biophys. Res. Commun.* **124**, 815–821
- Nakayama K., Ohkubo H., Hirose T., Inayama S. & Nakanishi S. (1984) *Nature (London)* **310**, 699–701
- Napier, M. A. & Blaine, E. H. (1984) *Annu. Rep. Med. Chem.* **19**, 253–262
- Napier, M. A., Dewey, R. S., Albers-Schonberg G., Bennett, C. D., Rodkey, J. A., Marsh, E. A., Whinnery M., Seymann, A. A. & Blaine, E. H. (1984) *Biochem. Biophys. Res. Commun.* **120**, 981–988
- Nemer M., Chamberland M., Sirois D., Argentin S., Drouin, J., Dixon, R. A. F., Zivin, R. A. & Condra, J. H. (1984) *Nature (London)* **312**, 654–656
- Nordheim A. & Rich A. (1983) *Nature (London)* **303**, 674–679
- Oikawa S., Imai M., Veno A., Tanaka S., Noguchi T., Nakazato H., Kangawa K., Fukuda A. & Matsuo H. (1984) *Nature (London)* **309**, 724–726
- Palade, G. E. (1961) *Anat. Rec.* **139**, 262
- Perlman D. & Halverson, H. O. (1983) *J. Mol. Biol.* **167**, 391–409
- Schenk, D. B., Johnson, L. K., Schwartz K., Sista H., Scarborough, R. M. & Lewicki, J. A. (1985) *Biochem. Biophys. Res. Commun.* **127**, 433–442
- Seidah, N. G., Lazure C., Chretien M., Thibault G., Garcia, R., Cantin M., Genest J., Nutt, R. F., Brady, S. F., Lyle, T. A., Palaveda, W. J., Colton, C. D., Ciccarone, T. M. & Veber, D. F. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2640–2644
- Seidman, C. E., Duby, A. D., Choi E., Graham, R. M., Harber E., Honey C., Smith, J. A. & Seidman, J. G. (1984a) *Science* **225**, 324–326
- Seidman, C. E., Bloch, K. D., Klein, K. A., Smith, J. A. & Seidman, J. G. (1984b) *Science* **226**, 1206–1209
- Slighton, J. L., Blechl, A. E. & Smithies O. (1980) *Cell* **21**, 627–638
- Stoeckert, C. J., Collins, F. S. & Weissman, S. M. (1984) *Nucleic Acids Res.* **12**, 4469–4479
- Tanaka I., Misono, K. S. & Inagami T. (1984) *Biochem. Biophys. Res. Commun.* **124**, 663–671
- Thibault G., Garcia R., Cantin M. & Genest J. (1983a) *Hypertension* **5** Suppl. 1, 75–80
- Thibault G., Garcia R., Seidah, N. G., Lazure C., Cantin M., Chretien M. & Genest J. (1983b) *FEBS Lett.* **164**, 286–290
- Thibault G., Garcia R., Cantin M., Genest J., Lazure, C., Seidah, N. G. & Chretien M. (1984a) *FEBS Lett.* **167**, 352–356
- Thibault G., Garcia R., Carrier F., Seidah, N. G., Lazure C., Chretien M., Cantin M. & Genest J. (1984b) *Biochem. Biophys. Res. Commun.* **125**, 938–946
- Trippodo, N. D., Ghai, R. D., MacPhee, A. A. & Cole, F. E. (1984) *Biochem. Biophys. Res. Commun.* **119**, 282–288
- Trippodo, N. C., MacPhee, A. A. & Cole, F. E. (1983) *Hypertension* **5** Suppl. 1, 81–88
- Wakitani K., Currie, M. G., Geller, D. M. & Needleman P. (1985) *J. Lab. Clin. Med.* **105**, 349–352
- Wells, R. D., Migletta, J. J., Klysiak J., Larson, J. E., Stirdivant, S. M. & Zacharias W. (1982) *J. Biol. Chem.* **257**, 10166–10171
- Wilson, A. C., Carlson, S. S. & White, T. J. (1977) *Annu. Rev. Biochem.* **46**, 573–639
- Winquist, R. J., Faison, E. P., Waldman, S. A., Schwartz, K., Murad F. & Rapoport, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7661–7664
- Yamanaka M., Greenberg B., Johnson L., Seihamer J., Brewer M., Friedemann T., Miller J., Atlas S., Laragh, J., Lewicki J. & Fiddes J. (1984) *Nature (London)* **309**, 719–722
- Yip, C. C., Laing, L. P. & Flynn, T. G. (1985) *J. Biol. Chem.* **260**, 8229–8232
- Zivin, R. A., Condra, J. H., Dixon, R. A. F., Seidah, N. G., Chretien M., Nemer M., Chamberland M. & Drouin J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6325–6329