acid overlap with the *S*-acyl fatty acid synthase thioesterase from the uropygial gland of mallard duck [A. J. Poulose, L. Rogers, T. M. Cheesebrough, P. E. Kolattukudy, *J. Biol. Chem.* **260**, 15953 (1985)] and a 38.1% identity over a 217–amino acid overlap with the medium-chain *S*-acyl fatty acid synthetase thioester hydrolase from rat mammary gland [J. Naggert, B. Williams, D. P. Cashman, S. Smith, *Biochem. J.* **243**, 597 (1987); R. Safford *et al.*, *Biochemistry* **26**, 1358 (1987)].
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21. Aqueous solutions of Er A and 6-deoxyEr A, 0.5 mg/ml, were prepared in citrate buffer (pH 2.2), incubated at 37°C, and sampled at regular intervals. Samples and standards were analyzed and compared by TLC. After 2 min, the Er A solution had been converted (>95%) to biologically inactive anhydroEr. By comparison, 6-deoxyEr A displayed a half-life of approximately 6 hours under the same conditions.
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23. The strains were *Staphylococcus aureus, Streptococcus pyogenes*, and *Streptococcus pneumoniae* (R. N. Swanson *et al.*, unpublished data).
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A Genetic Tool Used to Identify Thioredoxin as a Mediator of a Growth Inhibitory Signal

LOUIS P. DEISS AND ADI KIMCHI*

Loss of sensitivity to growth inhibitory polypeptides is likely to be one of the events that participates in the formation of some tumors and might be caused by inactivation or loss of the genetic elements that transduce these extracellular signals. The isolation of such a gene was achieved by randomly inactivating genes by an anti-sense complementary DNA expression library followed by direct selection for growth in the presence of an inhibitory polypeptide. Thus, a gene whose inactivation conveyed growth resistance to interferon-γ (IFN-γ) was isolated. Sequence analysis showed complete identity with human thioredoxin, a dithiol reducing agent, implicated here in the IFN-γ–mediated growth arrest of HeLa cells.

TUMOR FORMATION IS OFTEN A MULtistep process in which many genetic alterations can be detected, including activation of proto-oncogenes (dominant alterations) as well as inactivation of tumor suppressor genes (recessive alterations) (*1*). Only a few tumor suppressor genes have been characterized, including *Rb*, *p53*, *DCC*, *NM23*, and *ras* suppressors (*2*), because there is no rapid selection method. To address this problem, we have developed a genetic tool for the rapid isolation of genes that inhibit proliferation in a specified restrictive environment. These genes would be logical candidates to test as possible tumor suppressor genes. We refer to this technique as the Technical Knock Out (TKO) selection; it is based on the random inactivation of genes via a cDNA library cloned into an anti-sense expression vector. The assumption is that the specific inactivation of a growth inhibitory gene would convey growth advantage in a specific restrictive environment. This growth advantage is the powerful forward selection that is used for isolation of the desired inactivation event from all the other random inactivation events.

Molecules capable of inhibiting cellular proliferation in cell culture and in the context of the entire organism include several structurally unrelated families of polypeptides, such as the interferon family [type I IFN (α and β) and type II IFN (IFN-γ)], tumor necrosis factors (TNFs) (α and β), the transforming growth factor–β (TGF-β) family, amphiregulin and oncostatin M (*3*). Some of these growth inhibitors, such as the IFNs and TGF-β, operate in a paracrine or an autocrine manner, especially in limiting the proliferation of differentiating tissues (*4*). In some hematopoietic tumors, the autocrine loop for type I IFN is inactivated by the deletion of the entire type I IFN gene cluster (*5*). The antiproliferative effects of IFNs, TGF-β, and TNFs were attributed in some cells to their ability to suppress c-*myc* (*6–9*). Furthermore, the failure of IFN to reduce c-*myc* expression in resistant tumor cells was complemented by fusion with sensitive cells, suggesting that it could result from a recessive genetic lesion that leads to neoplasia (*9*). We have used the growth inhibition of HeLa cells by IFN-γ as a model system for testing the TKO selection and for the isolation of a putative growth suppressor gene. IFN-γ also has a role in vivo as a major immune regulator, including anti-tumor regulation (*10, 11*). We used human recombinant IFN-γ, purified to homogeneity, in these studies (*8*).

The TKO selection requires the transfection of a large number of cells with a transcription unit that promotes high amounts of expression of an anti-sense cDNA library in the continuous presence of IFN-γ. We chose the Epstein-Barr virus (EBV) episomal vector (*12*) to transduce the cDNA library because it efficiently transfects human cells. We were able to reproducibly obtain stable transfection rate for HeLa cells of 2 to 10% by introducing this vector through the standard calcium phosphate transfection technique (*13*). The episomal vector can be mechanically separated from the bulk of the human chromosomal DNA by Hirt extraction (*14*) and used to transform bacteria. This property allows the rapid isolation and identification of the cloned anti-sense cDNA contained in any selected cell. In addition, the episomal vector contains a hygromycin B–resistance marker.

Three different EBV-based expression vectors that could drive the transcription of the chloramphenicol acetyl transferase gene (*CAT*) were transfected into HeLa cells and selected for resistance to hygromycin B. The pTKO1-CAT and pTKO2-CAT contain the simian virus 40 (SV40) early promoter (Fig. 1). The pTKO1-CAT also includes an IFN response element (IRS) (*15*), cloned downstream of the *CAT* gene, to increase expression in IFN-treated cells. The pTKO3-CAT contains the murine sarcoma virus long-terminal repeat (MSV LTR) promoter. Pools of hygromycin B–resistant cells consisting of more than 10^4 independent clones were generated and treated with IFN-γ.

Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, Israel.

*To whom correspondence should be addressed.

Steady-state expression of CAT mRNA from pTKO1-CAT was stimulated both by IFN-γ treatment and cell confluence, which occurred 4 days after cells were plated (Fig. 2). Expression from the other two constructs, pTKO2-CAT and pTKO3-CAT, was inhibited by either IFN-γ or confluence. The same results were obtained in a second set of transfections in which the dihydrofolate reductase gene (*DHFR*) was expressed from pTKO1 instead of *CAT* (*16*). Thus, the pTKO1 construct was chosen to express the cDNA library. The presence of the IRS in the pTKO1 vector will also increase the probability of hitting elements that are unique to the growth inhibitory pathways of IFN, since we have found that in many IFN growth-resistant cell lines and variants the IRS-regulated genes continued to be normally induced by IFN (*6, 9*).

The cDNA library was generated from a mixture of RNAs harvested before and at 1, 2, 4, 12, 24, and 48 hours after IFN-γ treatment (200 units/ml), so that both constitutive and IFN-induced mRNA transcripts were included. Double-stranded cDNA was generated by the method of Gubler and Hoffman (*17*). The cDNA was made directional by linker addition, as described by Meissner *et al.* (*18*). The entire cDNA population was inserted into pTKO1 between Hind III and Bgl II restriction sites in the anti-sense orientation (*19*). This anti-sense library, consisting of approximately 10^5 independent clones, was introduced into 5×10^7 IFN-sensitive HeLa cells. A fraction of the transfectants was selected with hygromycin B alone to determine in parallel the rate of stable transfection. This titration indicated a transfection rate of 7%, resulting in the generation of 3.4×10^5 stable transformants. The majority of the transfected cells was seeded at a cell density of 1500 cells per square centimeter selected with hygromycin B (200 μg/ml) and IFN-γ (200 units/ml). The initial cell density and the concentrations of hygromycin B and IFN-γ were calibrated in pilot transfections with the control vector pTKO1-DHFR (Fig. 1) and were found to strongly influence survival of false positives (*16*). During the selection in the conditions described above, the cells were growth-arrested after 2 days, followed by massive cell death a few days later. After 28 days of selection, there was a difference in the number and size of colonies in the plates that were transfected with the library (50 to 200 per plate) compared to the plates transfected with the control vector on which only 10 to 20 small colonies appeared. Selective media was changed twice each week during the selection. The colonies in the library-transfected plates were pooled. The anti-sense vectors contained in these cells were isolated by the method of Hirt (*14*), cleaved with the restriction enzyme Dpn I, which cleaves only input nonreplicated DNA (*20*), and transferred into bacteria. The Dpn I step was included to ensure that only episomes that had replicated in the HeLa cells were transferred into bacteria. This produced 20 independent bacterial colonies. All of these colonies contained a similar insert of approximately 350 bp in size, as determined by restriction mapping (*16*).

When the insert contained in this vector was subcloned and sequenced, the sequence was found to match that of human thiore-

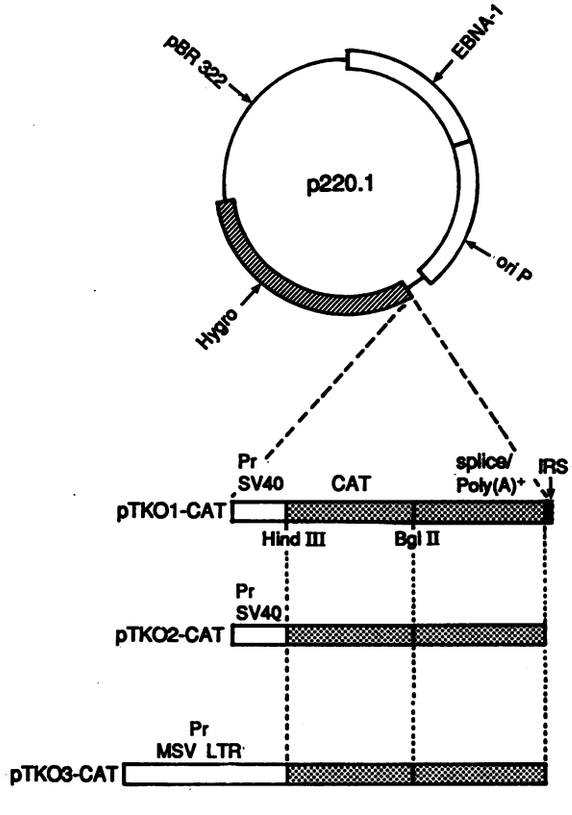

Fig. 1. Construction of EBV-derived expression vectors. The transcription casettes were cloned into the EBV episomal vector p220.1 (*12*). The p220.1 vector contains the Epstein-Barr virus nuclear antigen type 1 coding sequence (EBNA-1), the episomal origin of replication (ori P), a hygromycin B resistance marker (hygro), and the pBR322 sequences necessary for bacterial propagation. pTKO1-CAT was constructed with a transcription cassette derived from pSV2DHFR, which contains the SV40 early promoter, the coding sequences of the dihydrofolate reductase gene (*DHFR*) located between Hind III and Bgl II restriction sites, and a splice and polyadenylation signal [poly(A)$^+$] derived from SV40 (*28*). In addition, a multimer of the human IFN response element (IRS) (*15*) was inserted distally to the polyadenylation signal. The CAT coding region was derived from pGEMCATO (Hind III to Bam HI fragment) (*15*) and was cloned between the Hind III and Bgl II sites, replacing the *DHFR* coding sequences. The plasmid version containing the *DHFR* gene is denoted pTKO1-DHFR. pTKO2-CAT is similar to pTKO1-CAT, except that the IRS element was removed. pTKO3-CAT is similar to pTKO2-CAT, except that the SV40 promoter was replaced by the MSV LTR (*29*). With the use of DNA polymerase I (Klenow) and ligase, Eco RI–Bam HI fragment of pSV2 DHFR (*28*) was joined to a Hinc II–Bam HI fragment that contained six repeats of the oligomer of the human IRS sequence (*15*). This version of pSV2 DHFR had the Pvu II site converted to a Sal I site. The Hind III–Xba I fragment of this construct, containing the *DHFR* gene, the SV40 splice, and polyadenylation signal, as well as the IRS, was joined to the episomal vector p220.1 between Xba I and Hind III (*12*). Subsequently, a Sal I–Bgl II fragment from pSV2DHFR, which contains the SV40 early promoter, was cloned into this construct between Hind III and Bgl II. Finally, the Hind III site upstream of the promoter was removed by a partial Hind III digestion followed by DNA polymerase I (Klenow) treatment and ligation. These steps generate pTKO1-DHFR, which contains the DHFR gene in the transcription casette. pTKO2-DHFR was generated by Bam HI cleavage and ligation of pTKO1-DHFR, which removes the IRS multimer but otherwise leaves the plasmid intact.

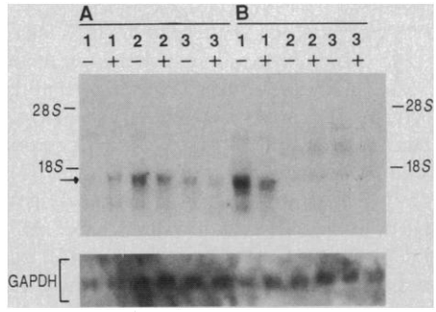

Fig. 2. The effects of IFN-γ on the mRNA expression from different EBV-derived expression vectors. Northern analysis of RNA from pools of cells stably transfected with pTKO1-CAT, pTKO2-CAT, and pTKO3-CAT corresponding to samples 1, 2, and 3, respectively (details of the plasmids in Fig. 1). We seeded 2×10^4 cells per square centimeter in the absence (−) or presence (+) of 200 units/ml of IFN-γ. Total RNA was extracted by the lithium chloride procedure (*8*) at (**A**) 15 hours or (**B**) 4 days after treatment. Total RNA (15 μg) was loaded in each lane. Electrophoresis and blotting were performed as previously described (*8*). The blots were hybridized with the Hind III–Bam HI fragment containing the *CAT* gene described in Fig. 1. The 18*S* and 28*S* ribosomal RNAs are indicated. The predicted position of the *CAT* mRNA is indicated by an arrow. The lower panels show the same blot hybridized with a glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe to correct for the amount of RNA loaded on each slot.

doxin (*21, 22*). The anti-sense orientation of the cDNA was verified both by restriction and sequence analysis (*16*). The double-stranded DNA containing this sequence was used as a probe in Northern (RNA) analysis (Fig. 3). It hybridized to the expected broad band in the 500 to 600 base range in nontransfected HeLa epithelial cells and Ha-Cat human keratinocyte cells (*23*) (Fig. 3). In stable transfectants carrying the anti-sense thioredoxin construct, the composite anti-sense transcript was detected. It consisted of 350 bases of the thioredoxin cDNA and 800 bases of sequences derived from the expression casette. In addition, the endogenous mRNA was detected (Fig. 3).

Next, the functional test was performed to determine if such an isolated anti-sense construct could individually convert HeLa cells from IFN-γ sensitivity to IFN-γ resistance, as defined by the double-selection conditions that were initially used to select the IFN-γ–resistant cells. The anti-sense thioredoxin construct pTKO1-ATx, as well as control vector pTKO1-DHFR, were introduced into cells, and pools of hygromycin B–resistant cells were generated (more than 10^4 clones per pool). The two pools were then cultured in the presence of both hygromycin B and IFN-γ. The presence of pTKO1-ATx (expressing anti-sense thioredoxin) significantly decreased the sensitivity of the transfected cells to growth inhibition (Fig. 4). This is reflected by a pronounced increase (by a factor of 100) in the number of colonies growing in the presence of IFN-γ. This bioassay was repeated eight times and always gave a similar increase in the number and size of colonies, as shown in Fig. 4. Thus, anti-sense thioredoxin was able to modulate the sensitivity of HeLa cells to IFN-mediated growth arrest.

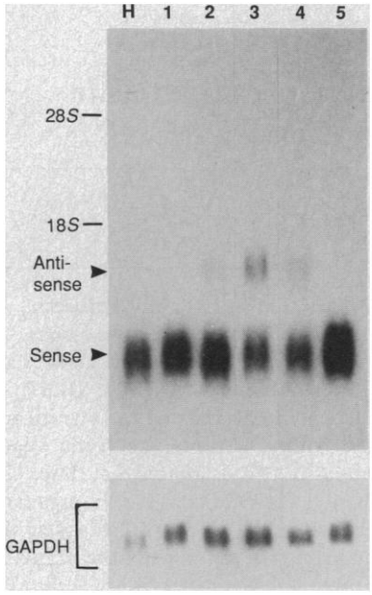

Fig. 3. Northern analysis of RNA from pTKO1-ATx–transfected cells. Total RNA was extracted from untransfected HeLa cells (H), Ha-Cat keratinocytes (lane 5), or pools of HeLa cells transfected with either control vector pTKO1-DHFR (lane 1) or the anti-thioredoxin construct pTKO1-ATx (lanes 2 to 4). The cells in lanes 1 and 2 were selected only with hygromycin B (200 μg/ml). Portions of the cells in lane 2 were then doubly selected [hygromycin B (200 μg/ml) and IFN-γ (200 units/ml)] for an additional 4-week period (lane 3), and a sample was washed in IFN-γ–free media and cultured with hygromycin B alone (200 μg/ml) for an additional 8-week period (lane 4). Before RNA harvest, the cells were grown without any selection for 1 week, in order to measure the amount of RNA independent of any direct influence of hygromycin B or IFN-γ on mRNA expression. Total RNA (15 μg) was loaded on each lane. The double-stranded thioredoxin cDNA insert, which hybridizes to both the sense and anti-sense mRNA, was used as a probe. The position of the 18*S* and 28*S* ribosomal RNAs, as well as the expected size of the sense and anti-sense mRNA, are indicated. The lower panel shows the same blot hybridized with a GAPDH probe to correct for the amount of RNA loaded in each slot.

The bioassay results indicate that thioredoxin is involved in the IFN-mediated arrest of HeLa cells. However, only a portion of the stably anti-sense thioredoxin-transfected cells grew in the presence of IFN-γ (Fig. 4). Since the mRNA for thioredoxin is abundant in HeLa cells (Fig. 3, lane H), it is expected that only the fraction of transfected cells that express high concentrations of anti-sense thioredoxin will grow. The copy number of the episomal vector is not absolutely fixed, and therefore there will be a variation in the copy number of the vector in each cell, resulting in variation in the amounts of expression. If these assumptions are true, the anti-sense thioredoxin (pTKO1-ATx)–transfected cells selected for IFN-γ resistance should show a higher amount of anti-sense thioredoxin mRNA than the population selected only with hygromycin B. This hypothesis was tested by examining the amounts of expression of anti-sense thioredoxin mRNA before and after long-term treatment with IFN-γ (Fig. 3). A strong selection was observed for higher anti-sense thioredoxin expression in the IFN-γ–selected cells (compare lane 2 to lane 3 in Fig. 3). We removed IFN-γ 1 week before RNA extraction to eliminate any direct effect of the IFN-γ on expression of mRNA from the vector. Furthermore, when IFN-γ was omitted for several weeks, there was a drift in the amounts of expression back to the concentrations observed before IFN-γ selection (compare lanes 3 to 4). Again, this is probably a result of the vector being episomal and not integrated. These two observations could explain why only a fraction of the transfected cells show IFN-γ resistance.

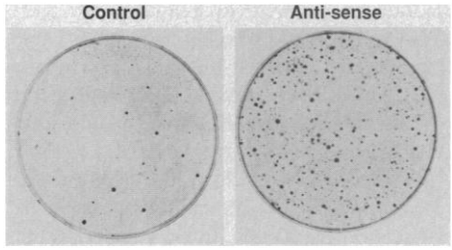

Fig. 4. Biological assay of the isolated anti-thioredoxin construct. HeLa cells were transfected with either the control vector pTKO1-DHFR or with the isolated construct pTKO1-ATx, which contained a thioredoxin gene segment in the anti-sense orientation. Pools of more than 10^4 independent clones from both transfections were first selected with hygromycin B to generate pure populations of stably transfected cells. These pools were plated in a 9-cm plate (100,000 cells per plate) and doubly selected with IFN-γ (200 units/ml) and hygromycin B (200 μg/ml). After 21 days, the plates were fixed with ethanol and stained with Giemsa. In the absence of IFN-γ, these plates reach confluency after about 1 week.

We conclude that the TKO selection does work and can be used to identify genes whose expression below some threshold amount might be rate-limiting in transducing extracellular negative signals. Thus, this technique could identify recessive alterations that relieve cells from some growth constraints, such as is expected for a subset of tumor suppressor genes. We have used this selection and identified thioredoxin as a gene that can modulate the IFN-γ–mediated growth arrest of HeLa cells. The fact that only this gene was recovered (out of a screen of 20 bacterial colonies) suggests that thioredoxin is the most abundant gene that can be isolated by this technique in this particular system and with this specific library.

Although thioredoxin acts as a powerful reducing agent (*24*), the mechanism by which it acts in the antiproliferative mode of action of IFN-γ is not known yet. Understanding the mechanism will require the construction of transfectants with stable integration of plasmids that produce continuous high-level expression of anti-sense thioredoxin mRNA. So far, the antiproliferative action of IFN-γ has been attributed to down-regulation of *c-myc* in some cell systems (*8*) and to the production of free radicals, depletion of nicotinamide-adenine dinucleotide pools, and depletion of extracellular tryptophan in other systems (*25*). Thioredoxin could be involved in coupling receptor events to some of these effects through dithiol reduction of crucial proteins. Several proteins with central roles in signaling pathways are regulated by thioredoxin, including the glucocorticoid receptor, Fos/Jun heterodimers, and possibly phospholipase C type I (*26*). Thus, thioredoxin may act as a signal transducer or

modulator. Thioredoxin also has an extracellular function as a secreted form, displaying many of the activities of interleukin-1 (IL-1) (*22*). Alternatively, since IL-1 can induce the production of free radicals (*27*), it is possible that thioredoxin (acting like IL-1) and IFN-γ together could induce inhibitory concentrations of free radicals. The challenge now is to determine whether these effects, or others, are significant during the growth arrest of HeLa cells.

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19. The cDNA library was constructed from 10 μg of oligo(dT)-selected mRNA by the method of Gubler and Hoffman (*17*). The resulting dscDNA was methylated by Bam HI and Alu I methylases and ligated to the linker described by Meisner *et al.* (*18*). Briefly, the ligation to the polyadenylated end of the cDNA generates a Hind III site, whereas at the 5′ end of the cDNA a Bam HI site is generated. This was followed by cleavage with Bam HI and Hind III and purification on an S-400 spin column (Promega). The purified dscDNA was ligated, at a 3:1 ratio, to Hind III– and Bgl II–cleaved pTKO1, and the ligation mixture was used to transform a variant of HB101 (obtained from M. Oren). The pTKO1 was generated by digestion of pTKO1-DHFR with Hind III and Bgl II, and dephosphorylation followed by preparative gel electrophoresis. The HB101 was made competent by the method of D. Hanahan [*J. Mol. Biol.* **166**, 557 (1983)], except that the dimethyl sulfoxide step was omitted.
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Selective Activation of the B Natriuretic Peptide Receptor by C-Type Natriuretic Peptide (CNP)

KERRY J. KOLLER,* DAVID G. LOWE, GREGORY L. BENNETT, NAOTO MINAMINO, KENJI KANGAWA, HISAYUKI MATSUO, DAVID V. GOEDDEL

The natriuretic peptides are hormones that can stimulate natriuretic, diuretic, and vasorelaxant activity in vivo, presumably through the activation of two known cell surface receptor guanylyl cyclases (ANPR-A and ANPR-B). Although atrial natriuretic peptide (ANP) and, to a lesser extent, brain natriuretic peptide (BNP) are efficient activators of the ANPR-A guanylyl cyclase, neither hormone can significantly stimulate ANPR-B. A member of this hormone family, C-type natriuretic peptide (CNP), potently and selectively activated the human ANPR-B guanylyl cyclase. CNP does not increase guanosine 3′,5′-monophosphate accumulation in cells expressing human ANPR-A. The affinity of CNP for ANPR-B is 50- or 500-fold higher than ANP or BNP, respectively. This ligand-receptor pair may be involved in the regulation of fluid homeostasis by the central nervous system.

ANP WAS THE FIRST DESCRIBED member of a family of polypeptide hormones that regulates salt and water balance and blood pressure (*1*). Subsequent to the isolation and characterization of ANP, two related hormones have been described: BNP, isolated from both brain and heart (*2, 3*), and CNP, purified from porcine brain (*4*). Like ANP, both of these hormones can elicit vasorelaxant, natriuretic, and diuretic responses in chick and rat bioassay systems (*2, 4*). These natriuretic peptides share a common structural motif consisting of a 17–amino acid loop formed by an intramolecular disulfide linkage. Only 5 of the 17 amino acids in the ring differ among the three peptides isolated from the pig, whereas the NH_2- and COOH-terminals vary in both amino acid composition and length (*4*). Among different species, the structures of both ANP and CNP are highly conserved (*1, 5*), whereas the amino acid sequence of BNP varies as much as 50% (*2, 3, 6*).

The biological activities of the natriuretic peptides are thought to be mediated by intracellular accumulation of guanosine 3′,5′-monophosphate (cGMP) through the activation of particulate guanylyl cyclase (*1, 7, 8*). Molecular cloning studies have identified three distinct natriuretic peptide receptors. ANPR-A and ANPR-B (also called

K. J. Koller, D. G. Lowe, D. V. Goeddel, Department of Molecular Biology, Genentech, Inc., South San Francisco, CA 94080.
G. L. Bennett, Department of Immunology Research and Assay Technology, Genentech, Inc., South San Francisco, CA 94080.
N. Minamino and H. Matsuo, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565, Japan.
K. Kangawa, Department of Biochemistry, Miyazaki Medical College, Kihara, Kiyotake, Miyazaki 889-16, Japan.

*To whom correspondence should be addressed.