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9-17-2004

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**Abstract**

The blood flukes *Schistosoma mansoni* and *Schistosoma japonicum*inflict immense suffering as agents of human schistosomiasis. Previous investigations have found the nervous systems of these worms contain abundant immunoreactivity to antisera targeting invertebrate neuropeptide Fs (NPFs) as well as structurally similar neuropeptides of the mammalian neuropeptide Y (NPY) family. Here, cDNAs encoding NPF in these worms were identified, and the mature neuropeptides from the two species differed by only a single amino acid. Both neuropeptides feature the characteristics common among NPFs; they are 36 amino acids long with a carboxyl-terminal Gly-Arg-*X*-Arg-Phe-amide and Tyr residues at positions 10 and 17 from the carboxyl terminus. Synthetic *S. mansoni* NPF potently inhibits the forskolin-stimulated accumulation of cAMP in worm homogenates, with significant effects at $10^{-11}$ M. This is the first demonstration of an endogenous inhibition of cAMP by an NPF, and because this is the predominant pathway associated with vertebrate NPY family peptides, it demonstrates a conservation of downstream signaling pathways used by NPFs and NPY peptides.

**Disciplines**

Molecular Biology | Neuroscience and Neurobiology | Parasitology

**Comments**

This research was originally published in The Journal of Biological Chemistry. Judith E. Humphries, Michael J. Kimber, Yi-Wen Barton, Walter Hsu, Nikki J. Marks, Brett Greer, Pat Harriott, Aaron G. Maule and Tim A. Day. Structure and Bioactivity of Neuropeptide F from the Human Parasites Schistosoma mansoni and Schistosoma japonicum. *Journal of Biological Chemistry*. 2004; 279:39880–39885. © the American Society for Biochemistry and Molecular Biology.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 38, Issue of September 17, pp. 39880–39885, 2004
Printed in U.S.A.
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# Structure and Bioactivity of Neuropeptide F from the Human Parasites *Schistosoma mansoni* and *Schistosoma japonicum**

Received for publication, May 20, 2004, and in revised form, June 28, 2004
Published, JBC Papers in Press, June 30, 2004, DOI 10.1074/jbc.M405624200

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**The blood flukes *Schistosoma mansoni* and *Schistosoma japonicum* inflict immense suffering as agents of human schistosomiasis. Previous investigations have found the nervous systems of these worms contain abundant immunoreactivity to antisera targeting invertebrate neuropeptide Fs (NPFs) as well as structurally similar neuropeptides of the mammalian neuropeptide Y (NPY) family. Here, cDNAs encoding NPF in these worms were identified, and the mature neuropeptides from the two species differed by only a single amino acid. Both neuropeptides feature the characteristics common among NPFs; they are 36 amino acids long with a carboxyl-terminal Gly-Arg-*X*-Arg-Phe-amide and Tyr residues at positions 10 and 17 from the carboxyl terminus. Synthetic *S. mansoni* NPF potently inhibits the forskolin-stimulated accumulation of cAMP in worm homogenates, with significant effects at $10^{-11}$ M. This is the first demonstration of an endogenous inhibition of cAMP by an NPF, and because this is the predominant pathway associated with vertebrate NPY family peptides, it demonstrates a conservation of downstream signaling pathways used by NPFs and NPY peptides.**

Blood fluke parasites of the genus *Schistosoma* are the most important metazoan parasites of humans and are the etiological agents of schistosomiasis (bilharzia), which afflicts over 200 million people. Schistosomiasis ranks second, behind only malaria, in terms of its overall negative socio-economic and public health impact on the tropical and subtropical world, and it remains among the top five disease priorities of the World Health Organization (WHO)[1] (1). Control of the disease rests almost solely on chemotherapy using the anthelmintic praziquantel (2–4). Most disturbingly, the long term utility of praziquantel has been brought into question because of growing reports of infections not responding to the recommended dosage (5–8) and worms with decreased sensitivity (9, 10). WHO has accordingly identified research into the basic biology of schistosomes as a priority, with the hope of identifying targets for the next generation of antischistosomal drugs (1).

Schistosomes belong to the class Trematoda of the phylum Platyhelminthes (flatworms) and, as such, are among the simplest extant animals to display brain development with the concomitant distinction between central and peripheral neuronal elements. One distinct feature of platyhelminths and other early diverging phyla is a prominent peptidergic component within their nervous systems. One family of neuropeptides abundant among these early animals is the neuropeptide F (NPF) family (11–14). NPFs are 36–40-amino acid peptides featuring a carboxyl-terminal Gly-Arg-*X*-Arg-Phe-amide (GR*X*RF-$NH_2$) motif and tyrosine residues at positions 10 and 17 relative to the carboxyl terminus.

These characteristics are common to those of vertebrate NPY family peptides, 36 amino acid neuropeptides with R*X*R(F/Y)-$NH_2$ carboxyl termini, Tyr residues at positions 10 and 17 relative to the carboxyl termini, and prolines in a P*XX*P*XX*P motif near the amino terminus (15, 16). These structural similarities were the basis of the discovery of invertebrate NPFs, as they were first localized using antisera targeting the carboxyl termini of NPY family neuropeptides (11). The striking structural similarities between NPY family peptides and NPFs notwithstanding, the evolutionary relationship between these neuropeptides is not yet clear. One important aspect that remains unresolved is the relationship between the signaling pathways used by NPYs and NPFs. NPY family peptides act on a structurally diverse family of G protein-coupled receptors which, through $G\alpha_{i/o}$-mediated pathways, almost always produce an inhibition of cyclic AMP (cAMP) (15, 17, 18).

At present there is only indirect evidence for the occurrence of NPY-like neuropeptides such as NPF in schistosomes. The most compelling evidence is abundant and widespread immunoreactivity to antisera targeting pancreatic polypeptide, a member of the NPY family of peptides (19–21). Here we identify cDNAs encoding NPFs from the blood fluke parasites *Schistosoma mansoni* and *Schistosoma japonicum*. In addition, the biological activity of *S. mansoni* NPF is measured and discovered to activate potently the same signaling mechanism stimulated by NPY family peptides in vertebrates.

## EXPERIMENTAL PROCEDURES

*Parasite Material*—Schistosome-infected mice were supplied by The Biomedical Research Institute (Rockville, MD) and housed at Iowa State University. At ~8 weeks post-infection, adult male and female *S. mansoni* and *S. japonicum* were removed from the mesenteric vasculature, snap-frozen in liquid nitrogen, and stored at −80 °C.

*Peptide Extraction and Chromatography*—Approximately 10 g of frozen adult *S. mansoni* were homogenized to a fine powder by using a

* This work was supported by National Institutes of Health Grant R01-AI49162 (to T. A. D. and A. G. M.) and an Iowa Healthy Livestock Initiative grant (to T. A. D. and M. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY299474, AY533028, AY662954.*

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[1] The abbreviations used are: WHO, World Health Organization; DAP, distal adaptor primer; EST, expressed sequence tag; FaRP, FMRFamide-related peptide; HPLC, high performance liquid chromatography; NPF, neuropeptide F; NPY, neuropeptide Y; PAP, proximal adaptor primer; PTX, pertussis toxin; RACE, rapid amplification of cDNA ends; mxNPF, *M. expansa* NPF; smNPF, *S. mansoni* NPF.


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pestle and mortar. Acid ethanol (EtOH, 0.7 M HCl; 3:1 v/w) was added to allow the formation of a paste that was further diluted with acid ethanol (total, 8:1 v/w) and stored overnight at 4 °C with continuous mixing. The solution was centrifuged at 4000 × *g* for 30 min to remove microparticulates; ethanol was removed using a Rotavapor (R100, Buchi), and a final concentration of 0.1% trifluoroacetic acid was added. This was stored for 18 h at 4 °C, centrifuged for 1 h at 4000 × *g,* and the supernatant lyophilized.

The lyophilized extract was reconstituted in 4 ml of glacial acetic acid, centrifuged for 30 min at 4000 × *g*, and the supernatant loaded onto a Sephadex G-50 (fine) column and eluted with 2 M glacial acetic acid at a flow rate of 10 ml/h; fractions were collected every 15 min. Fraction aliquots (10 μl) were monitored by using a neuropeptide F radioimmunoassay that employed an antiserum raised against the carboxyl-terminal decapeptide (YFAIIGRPRF-$NH_2$) of *Moniezia expansa* NPF (11). The assay sensitivity was 17 fmol of NPF per assay tube.

NPF-immunopositive fractions were subjected to reverse phase HPLC using a Phenomenex C-18 column (24 × 0.49 cm) and eluted with a linear gradient of 0.1% trifluoroacetic acid/$CH_3CN$ and 0.1% trifluoroacetic acid/$H_2O$ from 0:1 to 3:2 in 60 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals with absorbance monitoring at λ214 and 280 nm. Synthetic smNPF and GYIRFamide were subjected to identical HPLC fractionation conditions.

*Cloning of Schistosome Neuropeptide F-encoding cDNAs*—Cloning efforts focused on an *S. japonicum* expressed sequence tag (EST) potentially encoding a partial neuropeptide F prepropeptide.[2] Total RNA was isolated from ~100 *S. mansoni* or *S. japonicum* adults by using TRI Reagent (Sigma) and used to make separate amplified cDNA templates for use in RACE reactions according to the protocol of Matz (22). Briefly, first strand cDNA synthesis was driven by the dT variant primer TRsa. After second strand synthesis, a pseudo-double-stranded adaptor containing distal adaptor primer (DAP) sequence and proximal adaptor primer (PAP) sequence was ligated to both the 5′ and 3′ ends of the cDNA. The adaptor-ligated cDNA was then purified by using QIAquick PCR purification kit (Qiagen) and amplified using the DAP and TRsa primers for 20 cycles under the following conditions: 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 2 min 30 s. All reagents, except primers, were provided in the Marathon cDNA amplification kit (BD Biosciences) and used according to the manufacturer's instructions.

Seven gene-specific primers (F1–F7) for the *S. japonicum* EST were designed for 3′-rapid amplification of cDNA ends (RACE). Primers F1–F6 were used in primary 3′-RACE reactions with both the *S. japonicum* and *S. mansoni* cDNA, in conjunction with a DAP-TRsa 3′ adaptor-specific primer. The Advantage 2 PCR kit (BD Biosciences) was used for these reactions, and the components are as follows: 5 μl of DAP-TRsa (10 μM), 1.0 μl of gene-specific primer (10 μM), 2.5 μl of cDNA, 5.0 μl of 10× Advantage 2 Buffer, 1.0 μl of dNTP mix (10 mM each), 1.0 μl of Advantage 2 Taq, and sterile distilled water to 50 μl. The cycle profile consisted of 30 cycles of 94 °C for 30 s, 65 °C for 2 min, and 72 °C for 1 min. Reactions were visualized on a 1% agarose gel containing 0.5 mg/ml ethidium bromide. Primary RACE products were subsequently diluted 1:50 with sterile distilled water and reamplified with the appropriate nested gene-specific primer (F2–F7) in combination with the nested 3′ adaptor-specific primer TRsa. The components of these reactions were as before except 1 μl of each diluted primary RACE reaction was used as template with 1 μl of TRsa (10 μM) and 1 μl of F2–F7 (10 μM). The reamplification cycle conditions consisted of 30 cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 1 min. Six gene-specific 5′-RACE primers (R1–R6) were also designed from the *S. japonicum* EST. Primers R1–R5 were used in primary 5′-RACE reactions with both the *S. japonicum* and *S. mansoni* cDNA in conjunction with the DAP primer, otherwise the components of this reaction were the same as for the primary 3′-RACE reactions. The cycle profile for the 5′-RACE consisted of 30 cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 1 min. Additional 5′-RACE reamplifications were carried out as for the 3′-RACE but using the appropriate nested gene-specific primer (R2–R6) and the nested adaptor primer PAP. The reamplification cycle profile was the same as for the primary 5′-RACE.

*Amplification of S. mansoni Genomic DNA—S. mansoni* genomic DNA was isolated from ~50 worm pairs using the DNeasy tissue kit (Qiagen). Based on the sequence information obtained through RACE reactions, specific primers NPFgenF2 and NPFA2R were designed to amplify a PCR fragment encompassing the open reading frame of the *S. mansoni* NPF-encoding gene. Approximately 40 ng of genomic DNA served as template in PCRs using the Advantage 2 PCR kit (BD Biosciences). The components of these reactions are as follows: 1 μl of each primer (10 μM), 40 ng of genomic DNA, 5.0 μl of 10× Advantage 2 Buffer, 1.0 μl of dNTP mix (10 mM each), 1.0 μl of Advantage 2 Taq, and sterile distilled water to 50 μl. PCR cycle conditions were 30 cycles of 94 °C for 30 s, 59 °C for 1 min, and 72 °C for 1 min. Reactions were visualized on a 1% agarose gel containing 0.5 mg/ml ethidium bromide.

*Cloning and Sequencing*—Discrete bands from RACE reactions and genomic DNA amplifications were gel-purified using MinElute gel extraction kit (Qiagen). The purified PCR products were cloned using TOPO TA cloning kit for sequencing (Invitrogen), and colonies were grown overnight in LB medium containing 100 mg/liter ampicillin. Plasmid DNA was extracted from the cultures by using QIAprep spin miniprep kit (Qiagen), and the presence of an insert of the expected size was confirmed by digestion with EcoRI (New England Biolabs). Plasmids were sequenced using M13 forward and reverse primers, and raw sequence data were edited by using Vector NTI software (Informax).

*cAMP Assays*—Adult *S. mansoni* (4–6 weeks post-infection) were washed twice with cold cAMP buffer containing 50 mM sucrose, 50 mM glycylglycine, 10 mM creatine phosphate, 2 mM $MgCl_2$, 0.5 mM isobutylmethylxanthine, 1 mM dithiothreitol, 0.02 mM EGTA, 5 units of creatine kinase, and 0.01% bovine serum albumin. The worms were kept on ice for 5 min and then homogenized on ice for 2 min with a Teflon homogenizer. This preparation was centrifuged at 1000 × *g* for 5 min, and the pellet that included cell debris was discarded. The supernatant was centrifuged at 40,000 × *g* for 30 min at 4 °C. The supernatant was discarded, and the pellet containing the cell membranes was resuspended in cAMP buffer with 0.1 mM ATP. This membrane preparation was used for the cAMP assay, with each reaction containing membranes from ~3 worms.

The membrane mixture was incubated with various concentrations of forskolin at 37 °C for 20 min to stimulate cAMP production. Forskolin was dissolved in $Me_2SO$ with a final concentration of <0.1% $Me_2SO$ in the reaction mixture, which had no measurable effect on cAMP. Concentrations of cAMP were determined using radioimmunoassay as described previously (23). The lowest detectable quantity of cAMP in this assay was 1.6 fmol per tube.

*Peptides*—The smNPF and the amino-terminally truncated analog smNPF-(13–36) were generated at a 100-μmol scale using a 433A peptide synthesizer (Applied Biosystems, UK) and Rink amide 4-methylbenzhydrylamine resin (24). Peptide elongation was effected using 2-(1*H*-benzotriazole-1-y)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole·$H_2O$/*N*,*N*-diisopropylethylamine (1:1:2 v/v/v) and a 10-fold molar excess of each $N^{\alpha}$-Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-protected amino acid. Peptides were cleaved from the resin by a 2-h incubation at room temperature in trifluoroacetic acid/thioanisole/triisopropylsilane/water (96:2:1:1 v/v) and then separated from the resin by filtration under pressure, precipitated with diethyl ether several times, and lyophilized. Peptide purity was analyzed by reverse phase HPLC by using a Phenomenex C5 column (25 × 0.46 cm) and eluted with a linear gradient of 0.05% trifluoroacetic acid/$CH_3CN$ and 0.05% trifluoroacetic acid/$H_2O$ from 1:49 to 3:2, flow rate 1 ml/min with detection at λ 214 nm. Peptide identity was verified by electrospray mass spectroscopy using a Finnigan LCQ Ion Trap Mass Spectrometer.

Porcine NPY (pNPY), pNPY-free acid (p$NPY_{FA}$), human PP (hPP), and porcine PYY (pPYY) were obtained from American Peptide Co. *Moniezia expansa* NPF (mxNPF) was synthesized by CloneStar-UK. The platyhelminth FMRFamide-related peptides (FaRPs) GYIRFamide, RYIRFamide, and YIRFamide were synthesized by Research Genetics/Invitrogen.

*Bioinformatics*—BLAST searches were performed at National Center for Biotechnology Information (NCBI). SignalP analysis was executed on The World Wide Web Prediction Server at the Center for Biological Sequence Analysis, with truncation set at the default of 70 amino-terminal amino acid residues. Alignments were produced with the ClustalW algorithm by Vector NTI software (Informax).

## RESULTS

The earliest indication of an NPF-like neuropeptide in schistosomes was the widespread immunoreactivity with antisera targeting NPY family peptides observed throughout the nervous system of schistosomes (19–21). Substantiating this, in *S. mansoni* acid ethanol extracts there is a fraction immunoreactive with *M. expansa* NPF (mxNPF) antiserum (Fig. 1). The mxNPF radioimmunoassay detected only a single peak in

[2] GenBank™ accession number BU776442 submitted by the Chinese National Human Genome Center, Shanghai, China.

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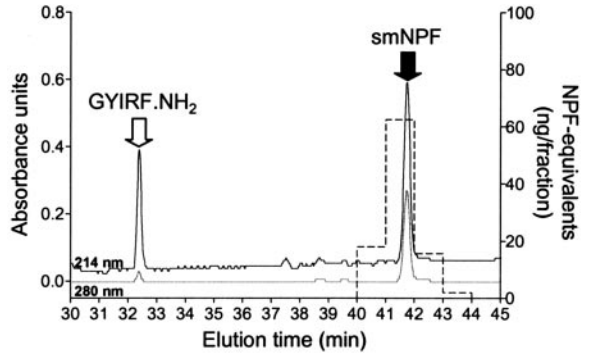


FIG. 1. **Chromatograph of mxNPF immunoreactivity in ethanol extracts of *S. mansoni* compared with the elution profile of synthetic flatworm neuropeptides.** The *dashed line* viewed against the right *y* axis indicates the results of radioimmunoassay by using an mxNPF antiserum (11) against an acid ethanol extract of mixed-sex, adult *S. mansoni*. The radioimmunoassay was performed against the fractions collected at 1-min intervals following fractionation on a Phenomenex C-18 analytical column eluted with a linear gradient of 0.1% trifluoroacetic acid/$CH_3CN$ (0–60% in 60 min). The major peak of mxNPF immunoreactivity in the schistosome sample is in the fraction collected in the 41st min. The *solid lines* show the 214- and 280-nm absorbance profiles for synthetic GYIRFamide and smNPF under the same conditions. Synthetic smNPF, the 36-amino acid amidated neuropeptide predicted by the smNPF cDNA, co-elutes with the major peak of mxNPF immunoreactivity recovered from the worm extracts.

*S. mansoni* following gel permeation and HPLC fractionation. The mxNPF-immunoreactive molecule co-eluted with synthetic mxNPF, indicating that the schistosome peptide was of similar size to mxNPF. Unfortunately, even in the extracts from over 10,000 schistosomes, the low levels of recovery of NPF-immunoreactivity coupled with the complex nature of whole *S. mansoni* extracts negated peptide isolation, sequencing, and mass analysis. Therefore, we turned to molecular approaches to identify the structure of the NPF-immunoreactive molecule.

*Identification of NPF-encoding cDNAs*—In order to identify schistosome NPF, we focused on the GRPRFGKR motif. This includes the highly conserved GRPRF carboxyl-terminal pentapeptide of known mature NPFs (see Fig. 3), the adjacent Gly which serves as a donor for the amidation reaction, and the common Lys-Arg dibasic cleavage site. Extensive attempts to identify *S. mansoni* NPF by using degenerate primers targeting this Arg-rich motif were without success. A BLAST search using the GRPRFGKR motif against the translated schistosome ESTs at the National Center for Biotechnology Information found that the *S. japonicum* EST SJECNA01 potentially encodes this sequence. In addition, the hypothetical protein contains a Tyr residue at position 10 relative to the carboxyl-terminal Phe, another feature common to all known NPFs. Sequence-specific primers targeting this EST were generated and used in RACE PCR with cDNA templates from both *S. japonicum* and *S. mansoni*.

To characterize the full-length transcript of the *S. japonicum* EST, we designed seven forward and six reverse gene-specific primers from the EST sequence. These primers were used in 3′- and 5′-RACE PCR with an amplified cDNA template generated from adult *S. japonicum* tissue. A 779-nucleotide transcript was identified that includes a 438-nucleotide open reading frame encoding a putative 146-amino acid protein.[3] The transcript also featured a polyadenylation signal (AATAAA) 22 nucleotides upstream of a poly(A)$^+$ tail.

To determine whether a homologous gene was expressed in *S. mansoni*, the *S. japonicum*-specific RACE primers were also used in 3′- and 5′-RACE PCR with an *S. mansoni* cDNA template, constructed in an identical manner to the *S. japonicum* cDNA. The *S. japonicum* primers successfully amplified from the *S. mansoni* cDNA template a 787-nucleotide transcript with a 441-nucleotide open reading frame encoding a putative 147-amino acid protein.[4] The transcript includes two conserved polyadenylation signals (AATAAA) 16 and 22 nucleotides, respectively, upstream of a poly(A)$^+$ tail.

The *S. japonicum* and *S. mansoni* NPF cDNAs each have a single open reading frame, but because each has multiple potential initiation codons, the length of the prepropeptide is not clear. For example, the *S. mansoni* cDNA could encode four different proteins ranging 147 to 127 amino acids, and the *S. japonicum* prepropeptide could range from 146 to 126 residues (Fig. 2). All known prepro-NPFs feature a signal peptide in the amino-terminal region adjacent to the mature peptide. SignalP analysis finds a signal peptide in each of the possible schistosome prepro-NPFs, with the exception of the potential 147-amino acid *S. mansoni* prepro-NPF. In mxNPF, the only other NPF identified thus far in a parasitic platyhelminth, the amino terminus of the 40-amino acid mature peptide is generated by cleavage at a single basic residue (11, 25). Both schistosome prepro-NPFs have a single basic residue at a site that would generate a 36-amino acid mature peptide (Fig. 2). The prepro-NPFs from both species also include conserved dibasic residues for carboxyl-terminal cleavage, resulting in propeptides with a carboxyl-terminal Gly residue required for the production of the α-amide group conserved among all known NPFs and NPY family neuropeptides. By assuming the earliest possible start site, the prepropeptides are 62% identical; the 36 amino acid mature neuropeptides are 97% identical between the 2 species, differing only by a single amino acid (Fig. 2 and Table I).

Both putative mature peptides have features that define NPFs (Fig. 3). The carboxyl-terminal GRPRF-$NH_2$ motif is conserved, which is not surprising because this was the basis of the identification of these peptides. The peptides also have Tyr residues at positions 10 and 17 relative to the carboxyl terminus, residues that are conserved in all NPFs and virtually all NPY family peptides (13, 16). The flatworm NPFs also include residues hypothesized to be present in the ancestral NPY family peptide (16): $Lys^{19}$, $Leu^{24}$, and $Leu^{30}$ (Fig. 3). NPY family peptides feature a highly conserved P*XXP*X*X*P motif in the amino-terminal half of the mature neuropeptides, but NPFs generally lack this feature. The amino-terminal half of schistosome NPFs is devoid of Pro residues but does possess a $Phe^{11}$ conserved among all but one of the flatworm and molluscan NPFs (Fig. 3).

To confirm that the 36-amino acid peptide predicted by the *S. mansoni* cDNA was the molecule originally identified by mxNPF immunoreactivity, the elution position of synthetic smNPF was compared with that of the mxNPF immunoreactivity in *S. mansoni* acid ethanol extracts. The immunoreactive fraction from *S. mansoni* co-eluted with synthetic smNPF (Fig. 1), supporting their structural similarity.

*S. mansoni Gene Organization*—The initial identification of NPF in a platyhelminth was from *M. expansa*. The gene encoding mxNPF features a phase 2 intron that is conserved among many NPY-encoding genes and that represents a possible link between invertebrate NPFs and vertebrate NPYs. To examine for the presence of introns, the *S. mansoni* gene was amplified from a genomic DNA template isolated from adult *S. mansoni* worms. The primers used in this PCR, NPFgenF2 and NPFA2R, were based on sequence obtained through 3′- and

[3] The *S. japonicum* NPF cDNA has GenBank™ accession number AY533028.

[4] The smNPF cDNA has GenBank™ accession number AY299474.

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FIG. 2. **Predicted NPF prepropeptides from *S. mansoni* and *S. japonicum*.** Amino-terminal peptide flanking the mature neuropeptide is on the *top line*, the propeptide is in the middle, and the carboxyl-terminal flanking peptide is on the *bottom line*. The figure includes the amino acids that would be encoded by the longest possible open reading frame, but both contain more than one potential initiation codon. Those denoted with (●) *above the sequence* result in peptides containing a signal peptide as identified by SignalP; a peptide initiated at the first Met in the *S. mansoni* open reading frame (○) is predicted to lack a signal peptide. Regardless of which Met represents the beginning of the prepropeptide, SignalP identifies the same single cleavage site in each peptide, and the first residue of the cleaved peptide is denoted with (▼). The site of the second amino-terminal cleavage is identified at a conserved single basic residue (■). Also present are dibasic cleavage sites producing the carboxyl-terminal of the propeptides (□□). The span that includes the predicted mature peptides, in the *middle rows*, includes only a single amino acid difference. Identical residues are blocked in *black*, and similar residues are blocked in *gray*.

TABLE I
*Comparison of prepropeptides of S. japonicum NPF and smNPF*

| | Longest possible prepropeptide | Amino-terminal extension | Propeptide | Carboxyl-terminal extension |
|---|---|---|---|---|
| *S. mansoni* | 147 aa[a] | 63 aa | 37 aa | 47 aa |
| *S. japonicum* | 146 aa | 61 aa | 37 aa | 48 aa |
| % similar | 71% | 67% | 100% | 54% |
| % identical | 62% | 55% | 97% | 46% |

[a] aa, amino acids.

5′-RACE and were designed to amplify a 792-nucleotide region of cDNA including the entire putative open reading frame of the gene.[5] The resulting genomic amplicon was also 792 nucleotides in length and was confirmed upon sequencing to contain no introns.

*Cyclic AMP Inhibition*—The closest structural analogs of invertebrate NPFs are members of the NPY family of peptides that act upon a family of receptors collectively designated Y receptors (17, 18). All known Y receptors are G protein-coupled, and all can be linked to inhibition of adenylyl cyclase through pertussis toxin-sensitive G proteins, either $G\alpha_i$ or $G\alpha_o$. All of the Y receptor subgroups have been shown to inhibit the accumulation of forskolin-stimulated cAMP, such that the inhibition of adenylyl cyclase has been referred to as the universal signaling mechanism of Y receptors (26). Therefore, we hypothesized that smNPF could act on its cognate receptor to inhibit the accumulation of forskolin-stimulated cAMP in schistosome homogenates.

We found that forskolin-stimulated cAMP accumulation in the homogenates was very similar to previous reports (27). With 100 $\mu$M forskolin for 20 min, cAMP accumulation was almost 400% of basal, and at 10 $\mu$M it was 258 ± 23% (Fig. 4*A*). We used 10 $\mu$M forskolin for subsequent assays. Synthetic smNPF produced a potent inhibition, with a threshold at 10 pM and an $IC_{50}$ of 170 pM (Fig. 4*B*). A maximum effect was produced near 10 nM with 70% inhibition. The carboxyl-terminal 24 amino acids of smNPF (smNPF-(13–36)) was only slightly less potent, with an $IC_{50}$ of 370 pM, and as with the holopeptide, smNPF-(13–36) had a maximal effect of 70% inhibition. The smNPF-mediated inhibition of cAMP was blocked by preincubation with 100 ng/ml PTX, indicating that the effect of the neuropeptide is transduced by $G\alpha_i$ or $G\alpha_o$ (Fig. 5*A*). smNPF also inhibited cAMP accumulation in the absence of forskolin stimulation. However, because the level of cAMP in the unstimulated homogenates was variable and relatively low, the results were much more erratic. For example, 1 $\mu$M smNPF inhibited cAMP accumulation by over 60% in one experiment, but the average was 41 ± 19% in three experiments.

*M. expansa* NPF (mxNPF) also produced a concentration-dependent inhibition of cAMP accumulation, but it was orders of magnitude less potent (Fig. 4*B*). The threshold was 10 nM, the same concentration at which smNPF produced a maximal effect. mxNPF is overall only 25% identical and 44% similar to smNPF, but the similarity is concentrated in the carboxyl-terminal region, where the last five residues are identical (GRPRF-$NH_2$) and seven of the last nine are similar. However, the nine carboxyl-terminal residues of mxNPF could not mimic the smNPF effect on cAMP, producing no inhibition at concentrations as high as 10 $\mu$M.

The structurally similar vertebrate peptides porcine NPY (pNPY) and human pancreatic polypeptide (hPP) produced a concentration-dependent inhibition of cAMP accumulation in the schistosome homogenates, but were dramatically less potent than smNPF. The only NPY family peptide tested that did not produce significant inhibition at 10 $\mu$M was porcine peptide YY (pPYY). It is difficult to attribute the efficacy of these neuropeptides to any particular carboxyl-terminal motif, because the rank order of potency for smNPF and its analogs was smNPF ( . . . GRPRF-$NH_2$) ≫ mxNPF ( . . . GRPRF-$NH_2$) > pNPY ( . . . TRQRY-$NH_2$) > hPP ( . . . TRPRY-$NH_2$) ≫ pPYY ( . . . TRQRY-$NH_2$), except that a GRPRF-$NH_2$ motif was more effective than the TRXRY-$NH_2$ motif (Table II). Although NPY was effective, NPY without a carboxyl-terminal amide was without effect, demonstrating a requirement for this carboxyl-terminal modification (Fig. 5*B*).

In addition to NPFs, another class of amidated peptides, designated FaRPs, have been found in worms of this phylum (13, 20, 28). Like NPFs, FaRPs possess a carboxyl-terminal RF-$NH_2$ signature. All of the platyhelminth FaRPs identified thus far (RYIRF-$NH_2$, GYIRF-$NH_2$, and YIRF-$NH_2$) were without effect on cAMP at concentrations as high as 10 $\mu$M (Fig. 5*C*).

## DISCUSSION

The cDNAs identified here in two species of schistosomes encode peptides that share the defining characteristics of NPFs. Furthermore, the neuropeptide sequence encoded by the *S. mansoni* cDNA has potent (threshold of 0.01 nM and $IC_{50}$ of 0.170 nM) inhibitory effects on cAMP accumulation in homogenates from these worms.

The cDNAs cannot, however, provide unequivocal identification of the mature neuropeptides; in the absence of sufficient tissue for biochemical purification, that certainty is lacking.

[5] The smNPF genomic sequence has GenBank™ accession number AY662954.

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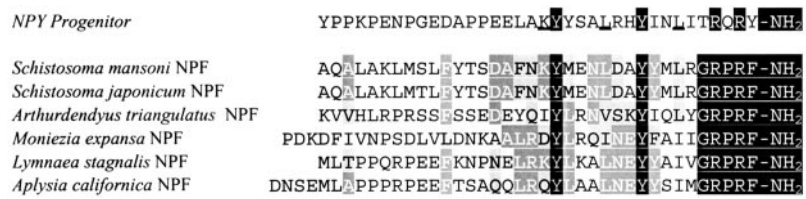


FIG. 3. **Alignment of schistosome NPFs with those of other platyhelminths, molluscs, and a putative NPY family peptide progenitor.** The lower grouping shows the flatworm and mollusc NPFs. The GRPRF-$NH_2$ motif is conserved, as are Tyr residues at positions 10 and 17 relative to the carboxyl terminus of the mature peptide. The alignment demonstrates that the conservation of the structure is much less in the amino-terminal half of the neuropeptides and greater in the carboxyl-terminal half, culminating in the identical carboxyl-terminal pentapeptide amide. Identical residues are blocked in *black,* and similar residues are blocked in *gray*. Above the invertebrate neuropeptides is a postulated NPY family peptide progenitor (16). The residues conserved between this peptide and the schistosome NPFs are *underlined*, and they include three residues not seen in all NPFs: $Lys^{19}$, $Leu^{24}$, and $Leu^{30}$.

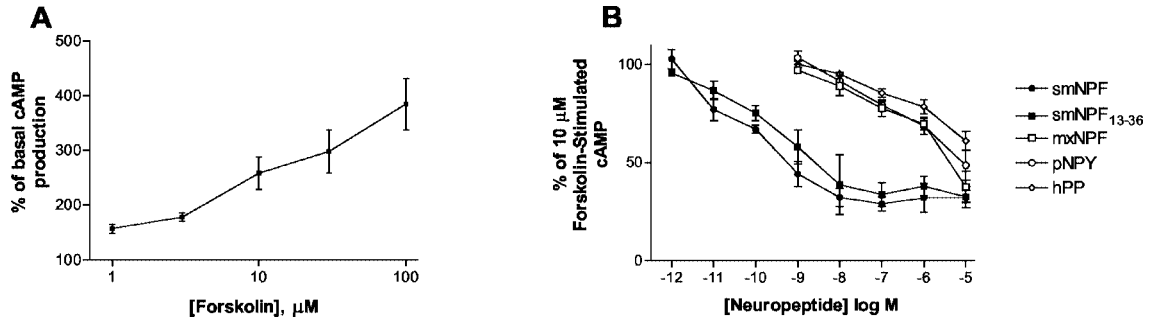


FIG. 4. **Concentration-dependent effects of forskolin and neuropeptides on cAMP levels.** *A*, forskolin stimulation of cAMP accumulation in *S. mansoni* homogenates. *B*, concentration dependence of its inhibition by synthetic smNPF and similar neuropeptides. The forskolin stimulation of cAMP in schistosomes has been reported previously (27, 37), and the results obtained here are very similar. If the forskolin incubations included smNPF, the accumulation of cAMP in the homogenates was inhibited in a concentration-dependent fashion, with a maximum inhibition of almost 70% of the stimulated cAMP. Synthetic schistosome peptides were the most potent by a wide margin, but other structurally similar peptides also produced a concentration-dependent inhibition of cAMP. For each data point, at least four different samples were tested on at least 2 different days, and the points displayed are the mean ± S.E.

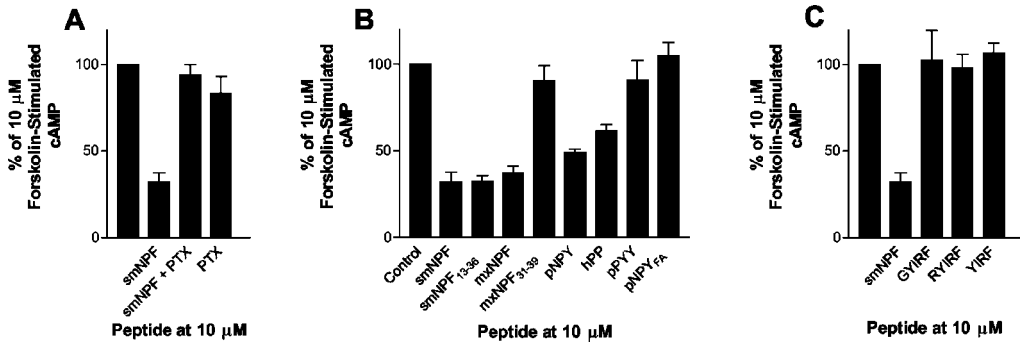


FIG. 5. **Inhibition of forskolin-stimulated cAMP production by neuropeptides.** *A*, the smNPF-mediated inhibition is blocked by preincubation of the homogenate with PTX (100 ng/ml), indicating a role for $G\alpha_{i/o}$ in the transduction of the smNPF. PTX alone did not have a significant effect. *B*, both smNPF and the carboxyl-terminal fragment smNPF-(13–36) inhibited cAMP accumulation. However, although mxNPF was effective, the carboxyl-terminal fragment mxNPF-(31–39) was without significant effect. Absence of the carboxyl-terminal amide, as seen in pNPY free acid ($pNPY_{FA}$), abolished the inhibitory effects demonstrating that the effect measured here is mediated by a receptor selective for amidated neuropeptides. *C*, the effect of smNPF on cAMP is not mimicked by any of the platyhelminth FMRFamide-related peptides. All the data are from at least four different samples tested on at least 2 different days, and displayed are the mean ± S.E.

However, a number of considerations suggest that the 36-amino acid mature peptide utilized for the biochemical studies here is the endogenous neuropeptide. First, size fractionation and HPLC did not distinguish between the endogenous mxNPF immunoreactive molecule in *S. mansoni* extracts and the synthetic 36-amino acid amidated smNPF. Second, if the schistosome prepro-NPFs are processed like the only other known parasitic flatworm NPF, the 36-amino acid mature peptide would result. For *M. expansa*, because both the cDNA and mature peptide have been identified (11, 25), it is known that cleavage at a single basic residue produces the amino terminus of the mature peptide. Both schistosome prepro-NPFs have a single basic residue where cleavage would produce the 36-amino acid mature peptide. Finally, the potency of smNPF36 on cAMP inhibition in schistosome homogenates strongly supports its designation as an endogenous signaling molecule in the worms.

The potency of smNPF inhibition of cAMP also leaves little doubt that this is an associated endogenous biochemical pathway in the worms. NPY family peptides exert their effects through the activation of a family of G protein-coupled receptors termed Y receptors. All five Y receptor subtypes produce an inhibition of adenylate cyclase, whereas two subtypes also produce elevation of cytoplasmic $Ca^{2+}$ (29–32). The inhibition of cAMP is so closely associated with Y receptors that it has been referred to as the universal signaling mechanism among them (18, 26). The inhibition of cAMP in schistosome homogenates by smNPF demonstrates a conservation of signaling between this NPF and NPY family peptides. The conservation of this signaling pathway has been suggested in the mollusc *Lym-*

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TABLE II
*Neuropeptides tested for their ability to inhibit the accumulation of forskolin-stimulated cAMP in S. mansoni homogenates*

| Peptide | Denotation in text | Amino acid sequence | $IC_{50}$ in cAMP assay |
|---|---|---|---|
| *S. mansoni* NPF | smNPF | AQALAKLMSLFYTSDAFNKYMENLDAYYMLRGRPRF-$NH_2$ | 170 pM |
| *S. mansoni* $NPF_{12–36}$ | $smNPF_{13–36}$ | TSDAFNKYMENLDAYYMLRGRPRF-$NH_2$ | 370 pM |
| *M. expansa* NPF | mxNPF | PDQDAIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRF-$NH_2$ | ≈1 μM |
| *M. expansa* $NPF_{31–39}$ | $mxNPF_{31–39}$ | FAIIGRPRF-$NH_2$ | NA[a] |
| Porcine neuropeptide Y | pNPY | YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-$NH_2$ | ≈1 μM |
| Porcine neuropeptide Y free acid | $pNPY_{FA}$ | YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY- | NA |
| Porcine neuropeptide $Y_{1–24}$ | $pNPY_{1–24}$ | YPSKPDNPGEDAPAEDLARYYSAL | NA |
| Human pancreatic polypeptide | hPP | APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-$NH_2$ | >1 μM |
| Porcine peptide YY | pPYY | YPAKPEAPGENASPEELSRYYASLRHYLNLVTRQRY-$NH_2$ | NA |
| Flatworm FaRP | | YIRF-$NH_2$ | NA |
| Flatworm FaRP | | GYIRF-$NH_2$ | NA |
| Flatworm FaRP | | RYIRF-$NH_2$ | NA |

[a] NA, no activity.

*naea stagnalis* (33) and *Drosophila* (34), where receptors responding to NPFs couple to cAMP inhibition in heterologous expression systems. However, this is the first demonstration of an endogenous inhibition of cAMP by an invertebrate NPF. These results therefore provide a more substantial link between NPF receptor-mediated signaling pathways in invertebrates and those of vertebrate NPY family peptides. These results also indicate that NPY/NPF signaling systems were present within more basal metazoa as platyhelminths are thought to have diverged prior to molluscs or arthropods (35).

The inhibition results with various NPFs and NPY family peptides show that the GRPRF-$NH_2$ carboxyl-terminal motif found in NPFs is more potent than is the TRQRY-$NH_2$ or TRPRY-$NH_2$ motif of many NPY family peptides. However, the ultimate efficacy of the neuropeptide is not determined solely by the carboxyl-terminal motif, because there is a dramatic difference in potency between smNPF and mxNPF, which both end in GRPRF-$NH_2$.

In total, the data reported here strengthen the hypothesis that invertebrate NPFs are related to NPY family peptides. First, the structures of *S. japonicum* NPF and smNPF add to the growing number of invertebrate neuropeptides with conspicuous similarities with NPY family peptides and the putative NPY family progenitor (16): 36 amino acid peptides with conserved Tyr residues at positions 10 and 17 relative to an R*X*R(F/Y)-$NH_2$ carboxyl terminus. Second, the schistosome peptides feature three additional residues ($Lys^{19}$, $Leu^{24}$, and $Leu^{30}$) that are common to the hypothesized NPY family progenitor, thereby providing an additional although weak link between flatworm NPFs and NPYs. Finally, as discussed above, the demonstration of continuity in the biochemical signaling pathway activated by smNPF and NPY family peptides supports the relationship between these neuropeptides.

The evidence is that NPF is the most abundant neuropeptide in these medically important parasites (19–21, 36) and that the peptide has extremely potent biochemical effects in the worm, which provides appeal to the NPF signaling system as a potential target for antischistosomal drugs. However, the conservation of structure with mammalian NPY family neuropeptides and the conservation of downstream biochemical signaling demonstrated here indicate similarities between the receptors for NPFs and vertebrate NPYs that could present a challenge in developing selective receptor pharmacophores. Regardless, the data presented here are a significant step toward understanding the biology of the most abundant neuropeptide in these parasites that continue to inflict an immense amount of suffering throughout the world.

*Acknowledgment*—We thank Dr. Fred Lewis at the Biomedical Research Institute for supplying schistosome-infected mice. We also thank Dr. Angela Mousley at Queen's University, Belfast, for help with the HPLC figure.

REFERENCES

1. Remme, J. H., Blas, E., Chitsulo, L., Desjeux, P. M., Engers, H. D., Kanyok, T. P., Kengeya Kayondo, J. F., Kioy, D. W., Kumaraswami, V., Lazdins, J. K., Nunn, P. P., Oduola, A., Ridley, R. G., Toure, Y. T., Zicker, F., and Morel, C. M. (2002) *Trends Parasitol.* **18,** 421–426
2. Cioli, D., and Pica-Mattoccia, L. (2003) *Parasitol. Res.* **90,** S3–S9
3. Andrews, P., Thomas, H., Pohlke, R., and Seubert, J. (1983) *Med. Res. Rev.* **3,** 147–200
4. Day, T. A., Bennett, J. L., and Pax, R. A. (1992) *Parasitol. Today* **8,** 342–344
5. Ismail, M., Botros, S., Metwally, A., William, S., Farghally, A., Tao, L. F., Day, T. A., and Bennett, J. L. (1999) *Am. J. Trop. Med. Hyg.* **60,** 932–935
6. Ismail, M. M., Taha, S. A., Farghaly, A. M., and el-Azony, A. S. (1994) *J. Egypt Soc. Parasitol.* **24,** 685–695
7. Bennett, J. L., Day, T. A., Liang, F. T., Ismail, M., and Farghaly, A. (1997) *Exp. Parasitol.* **87,** 260–267
8. Gryseels, B., Mbaye, A., De Vlas, S. J., Stelma, F. F., Guisse, F., Van Lieshout, L., Faye, D., Diop, M., Ly, A., Tchuem-Tchuente, L. A., Engels, D., and Polman, K. (2001) *Trop. Med. Int. Health* **6,** 864–873
9. William, S., Sabra, A., Ramzy, F., Mousa, M., Demerdash, Z., Bennett, J. L., Day, T. A., and Botros, S. (2001) *Int. J. Parasitol.* **31,** 1093–1100
10. William, S., Botros, S., Ismail, M., Farghally, A., Day, T. A., and Bennett, J. L. (2001) *Parasitology* **122,** 63–66
11. Maule, A. G., Shaw, C., Halton, D. W., Thim, L., Johnston, C. F., Fairweather, I., and Buchanan, K. D. (1991) *Parasitology* **102,** 309–316
12. Leung, P. S., Shaw, C., Maule, A. G., Thim, L., Johnston, C. F., and Irvine, G. B. (1992) *Regul. Pept.* **41,** 71–81
13. Day, T. A., and Maule, A. G. (1999) *Peptides* **20,** 999–1019
14. Dougan, P. M., Mair, G. R., Halton, D. W., Curry, W. J., Day, T. A., and Maule, A. G. (2002) *J. Comp. Neurol.* **454,** 58–64
15. Balasubramaniam, A. A. (1997) *Peptides* **18,** 445–457
16. Larhammar, D. (1996) *Regul. Pept.* **62,** 1–11
17. Larhammar, D. (1996) *Regul. Pept.* **65,** 165–174
18. Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998) *Pharmacol. Rev.* **50,** 143–150
19. Marks, N. J., Halton, D. W., Maule, A. G., Brennan, G. P., Shaw, C., Southgate, V. R., and Johnston, C. F. (1995) *Parasitology* **110,** 371–381
20. Skuce, P. J., Johnston, C. F., Fairweather, I., Halton, D. W., and Shaw, C. (1990) *Parasitology* **101,** 227–234
21. Skuce, P. J., Johnston, C. F., Fairweather, I., Halton, D. W., Shaw, C., and Buchanan, K. D. (1990) *Cell Tissue Res.* **261,** 573–581
22. Matz, M. (2002) *Methods Mol. Biol.* **183,** 3–18
23. Richards, J. S., Jonassen, J. A., Rolfes, A. I., Kersey, K., and Reichert, L. E., Jr. (1979) *Endocrinology* **104,** 765–773
24. Fields, G. B., and Noble, R. L. (1990) *Int. J. Pept. Protein Res.* **35,** 161–214
25. Mair, G. R., Halton, D. W., Shaw, C., and Maule, A. G. (2000) *Parasitology* **120,** 71–77
26. Michel, M. C. (1991) *Trends Pharmacol. Sci.* **12,** 389–394
27. Estey, S. J., and Mansour, T. E. (1987) *Mol. Biochem. Parasitol.* **26,** 47–59
28. Fairweather, I., Skuce, P. O., Brownlee, D. J., Moody, R. M., and Johnston, C. F. (1995) *Acta Biol. Hung.* **46,** 211–220
29. Mihara, S., Shigeri, Y., and Fujimoto, M. (1989) *FEBS Lett.* **259,** 79–82
30. Motulsky, H. J., and Michel, M. C. (1988) *Am. J. Physiol.* **255,** E880–E885
31. Aakerlund, L., Gether, U., Fuhlendorff, J., Schwartz, T. W., and Thastrup, O. (1990) *FEBS Lett.* **260,** 73–78
32. Fredholm, B. B., Jansen, I., and Edvinsson, L. (1985) *Acta Physiol. Scand.* **124,** 467–469
33. Tensen, C. P., Cox, K. J., Burke, J. F., Leurs, R., van der Schors, R. C., Geraerts, W. P., Vreugdenhil, E., and Heerikhuizen, H. (1998) *Eur. J. Neurosci.* **10,** 3409–3416
34. Garczynski, S. F., Brown, M. R., Shen, P., Murray, T. F., and Crim, J. W. (2002) *Peptides* **23,** 773–780
35. Olson, P. D., Cribb, T. H., Tkach, V. V., Bray, R. A., and Littlewood, D. T. (2003) *Int. J. Parasitol.* **33,** 733–755
36. Johnston, R. N., Shaw, C., Brennan, G. P., Maule, A. G., and Halton, D. W. (1995) *J. Comp. Neurol.* **357,** 76–84
37. Mansour, J. M., and Mansour, T. E. (1986) *Mol. Biochem. Parasitol.* **21,** 139–149

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# Structure and Bioactivity of Neuropeptide F from the Human Parasites *Schistosoma mansoni* and *Schistosoma japonicum*

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*J. Biol. Chem. 2004, 279:39880-39885.*
*doi: 10.1074/jbc.M405624200 originally published online June 30, 2004*

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