Neurospora crassa cDNA clones coding for a new member of the *ras* protein family

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Received 28 August 1990

A new member of the *ras* gene family was characterized from *Neurospora crassa* cDNA libraries. The clone designated *NC-ras* codes for a polypeptide containing 213 amino acids (M_r 24000). This polypeptide is 84% homologous to the *H-ras-1* domain comprising the first 80 amino acids and 60% homologous to the next 84 residues. The *NC-ras* polypeptide contains all the well-known sequences involved in the interaction with GTP/GDP, the recognition of the Y13-259 neutralizing antibody, the 'effector site' for interaction with GAP proteins, and the CAAX acylation motif in the COOH-terminal.

Neurospora crassa; *ras*-sequence; cDNA

1. INTRODUCTION

Ras, rho and *ypt* constitute a superfamily of regulator entities in eukaryotes [1,2]. Proto-oncogenic, normal, *ras* genes were described in human, rodent and chicken cells [3–7], as well as in *Drosophila* [8,9], *Dyctiostelium discoideum* [10], *Saccharomyces cerevisiae* [11] and *Schizosaccharomyces pombe* [12]. With the only exception of *S. cerevisiae*, where *ras* genes code for polypeptides of 40–41 kDa, the others code for 21–24 kDa polypeptides.

Although the physiological role of p21–24 *ras* proteins has not been elucidated, they seem to be involved in the control of cell proliferation and differentiation [13] through the interaction with a GTPase activating protein (GAP [14]. From a biochemical point of view, the *ras* family is closely related to the G-protein family. As occurs with this latter family, *ras* proteins have been shown to bind GTP and GDP, to have a GTPase activity and to be associated to the plasma membrane [15,16].

This paper describes the isolation of *Neurospora* cDNA clones coding for a protein of the *ras* family. These clones code for a 24 kDa protein containing all the characteristic domains of human, rat, *Dyctiostelium* and *Saccharomyces ras* proteins.

2. EXPERIMENTAL

2.1. *Strains and media*

Neurospora crassa wild-type Saint Lawrence 74 was grown in Vogel's liquid minimal medium [17] containing 2% sucrose (w/v) and 5 μg/ml D-biotin, for 48 h at 30°C with shaking.

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2.2. *cDNA libraries and DNA probes*

Two *Neurospora crassa* cDNA libraries were used. One of them, made in the λgt11 vector and containing inserts of about 300 bp, was kindly provided by Dr RajBandhari (Massachusetts Institute of Technology). The other, made in the λ-ZAP vector (Stratagene Cloning Systems, San Diego, CA, USA) and containing inserts longer than 1 kbp, was a gift of Dr M. Sach (Stanford University). Clones harboring *RAS1* and *RAD2 Saccharomyces cerevisiae* sequences were kindly provided by Dr M. Wigler (Cold Spring Harbor Laboratory).

2.3. *Preparation of DNA and RNA*

DNA from *Neurospora crassa* or from recombinant phages was prepared following the procedures of Blin and Stafford [18] and Yamamoto [19], respectively. Total *Neurospora crassa* RNA was obtained following the method of Reinert et al. [20] and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography [21].

2.4. *Labeling of DNA and hybridization*

DNA probes used for hybridization to DNA and RNA were labeled either by nick translation [22] or random priming [23].

DNA and RNA blottings were performed accordingly on Gene Screen membranes (New England Nuclear). For RNA blotting about 50 μg of total RNA or 10 μg of poly(A)$^+$ RNA were electrophoresed according to Lehrach et al. [24].

Hybridizations were carried out in the presence of 2× Denhardt solution, 100 μg/ml salmonsperm DNA, 1% sodium dodecylsulphate (w/v) and 10% dextran sulfate (w/v) at low stringency (T_m − 35: 5× SSC; 30% formamide; 42°C) or high stringency (T_m − 5: 0.5× SSC; 50% formamide; 42°C). Conditions for washings were 2× SSC, 0.1% SDS (w/v) and 50°C (T_m − 35) or 0.1× SSC, 0.1% SDS (w/v) and 60°C (T_m − 5).

2.5. *DNA sequencing*

It was performed by the dideoxy-chain termination method [25] after subcloning the restriction fragments into the M13mp18 and M13mp19 vectors [26]. Sequences were analyzed with the Pustell DNA/Protein Sequence Analysis Software (International Biotechnologies Inc., New Haven, CT, USA). *NC-ras* cDNA sequence has the EMBL Data Library accession number X53533 N. CRASSA NC-RAS CDNA.

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00145793/90/$3.50

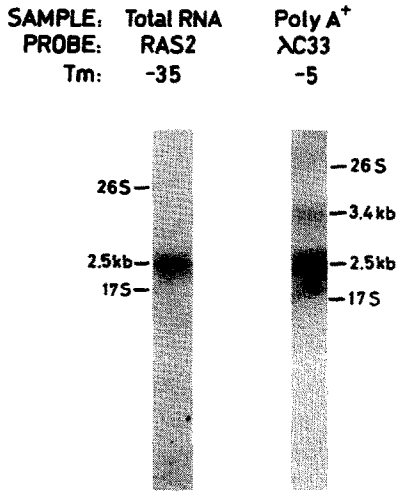

Fig. 1. Northern blots of *Neurospora crassa* total RNA or poly(A)$^+$ RNA. RNA was hybridized with the 1.2 kbp *Hpa*I restriction fragment from a *Saccharomyces cerevisiae RAS2* clone (T_m −35) or with the 553 bp *Eco*RI restriction fragment from *Neurospora crassa* λC33 clone (T_m −5), respectively. Mobilities of *Neurospora* 26 S and 17 S RNAs are also indicated, as well as those of 2.5 and 3.5 kb RNA species.

3. RESULTS AND DISCUSSION

A *Neurospora crassa* cDNA library made in the λgt11 vector was screened at low stringency (T_m −35) with a 1.2 kbp *Hpa*I restriction fragment from a *Saccharomyces cerevisiae RAS2* clone [11]. From 38 clones giving a strong hybridization signal, the clone designated λC33 (553 bp) was selected for further studies. The criterium for this selection was the capability of the corresponding insert to hybridize (T_m −5) to some mRNA species in Northern blots of *Neurospora* poly(A)$^+$ RNA. As can be seen in Fig. 1, the *S. cerevisiae RAS2 Hpa*I probe and the insert from the selected clone, could detect the presence of an mRNA species of about 2.5 kb. The λC33 probe also detected a minor component of about 3.4 kb.

The λC33 cloned insert was used to screen a *Neurospora crassa* cDNA library made in the λ-ZAP vector known to contain full-length representations of some RNAs. From about 70000 screened plaques, a clone containing an insert of about 1.9 kbp was selected. The insert corresponding to this clone, termed *NC-ras*, and that from λC33 were subcloned and sequenced. Fig. 2 shows a 1115 nucleotide partial sequence corresponding to *NC-ras* and the predicted amino acid sequence. λC33 sequence is included into *NC-ras* (residues 180–733). Several elements are evident in this sequence: (i) an open-reading frame for a protein containing 213 amino acids; (ii) the methionine start codon is contained into a consensus Kozak sequence (CCACAATGG) for initiation by eukaryotic ribosomes [27]; (iii) the sequence does not present a consensus polyadenylation signal suggesting that the 3′ non-coding region extends further downstream; and (iv) a 62 nucleotide long poly(dC-dA) tract is located 78 nucleotides downstream of the TGA stop codon, within the 3′ non-coding region. This tract was found in several mRNAs [28], including that encoded by the *BRL-ras*-related gene, a member of the mammalian *ras* superfamily [29] and it seems to be a transcription enhancer [30].

NC-ras codes for a polypeptide containing 213 amino acids with calculated molecular weight and isoelectric point of 24006.4 and 5.71, respectively. Fig. 3 shows

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   1 TTCACACAGGAAACAGCTATGACCATGATTACAATTCCGTGACGTTTGTCTGCACCGTTGCACGAGCGACTTAAACCATTTAATCATTTGAGGATTCCATTCAATCTTGCCCCCTCGTGT
 121 CGGCATCTCGACTTTTTCCATCCTTTTACATTTTACATCTTCTTTAATCAATAGCCACAATGGCCAACAAGTTCACAAGAGAATACAAGCTGGTGGTTGTCGGTGGCGGTGGTGTCGGTA
                                                    M  A  N  K  F  T  R  E  Y  K  L  V  V  V  G  G  G  G  V  G   20
 241 AATCGTGCCTTACCATCCAGCTAATCCAGGGTCACTTCCTTGACGAATATGATCCTACAATCGAGGATTCGTACCGCAAGCAATGCACCATCGACAATGAGGTCGCCCTTCTCGACATTC
       K  S  C  L  T  I  Q  L  I  Q  G  H  F  L  D  E  Y  D  P  T  I  E  D  S  Y  R  K  Q  C  T  I  D  N  E  V  A  L  L  D  I   60
 361 TAGATACCGCCGGCCAAGAAGAGTACTCCGCCATGCGCGAGCAGTACATGCGCACTGGTGAAGGCTTCCTGCTCGTTTTCGCCATCAACTCGAGGGAAAGCTTTGAGGAAATCAGAATCT
       L  D  T  A  G  Q  E  E  Y  S  A  M  R  E  Q  Y  M  R  T  G  E  G  F  L  L  V  F  A  I  N  S  R  E  S  F  E  E  I  R  I  100
 481 ACCAGCAACAGATTCTTAGGGTCAAGGACAGGGACTCGTTCCCCATGATCATCGTCGGAAACAAGTACGATTTGAGGGGAGAGCGCGTTGTTTCGGAACAGGAGGGACAAGCTCTTGCCG
       Y  Q  Q  Q  I  L  R  V  K  D  R  D  S  F  P  M  I  I  V  G  N  K  Y  D  L  R  G  E  R  V  V  S  E  Q  E  G  Q  A  L  A  140
 601 CCGAGTTCGGTACCAAGTACATCGAGACTTCCGCCAAGACACAACACAACGTTGAGAACGCTTTCTACGATCTCGTCCGCGAAATCAGGAAAGAGGACAAGAAGCTCGGCGAGAAGGTCG
       A  E  F  G  T  K  Y  I  E  T  S  A  K  T  Q  H  N  V  E  N  A  F  Y  D  L  V  R  E  I  R  K  E  D  K  K  L  G  E  K  V  180
 721 GCGGCACCTCATTCGCCAACAACAATGGGGCAGTTAAGCAGATGGACGTCGGTGACGAGGACGTCCAAGCAGGCTGCTGCGCCAAGTGCATAATGATGTGAATGATCGAAACCAACAGGG
       G  G  T  S  F  A  N  N  N  G  A  V  K  Q  M  D  V  G  D  E  D  V  Q  A  G  C  C  A  K  C  I  M  M  -                213
 841 GAAGAGCAAGCGGGGAGTTAAAAGAATTGGGACACGTTTGCAATGAAGAGATGGACGATCACACACACACACACACACACACACACACACACACACACACACACACACACACGCACAC
 961 ACGCACACAAAATCGACTTTATAGACGACATGGATGTGCAAACAACCGAAACACAAAAGCCATATCGGGACGGCCACGCCCATACCTTAGCTCACGTACGGTGACCAGTCGGGCTCAGGA
1081 CCACGGGACCAGCGAAGGAATATCGCGTTGAGAAA
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Fig. 2. Nucleotides and predicted amino acid sequences of *Neurospora crassa NC-ras*. Sequence of the λC33 clone corresponds to that between nucleotides 180 and 733. Underlined bases indicate positions of *Sal*I site, Kozak sequence, *Xba*I and *Hin*dIII sites, and the poly(dC-dA) sequence.

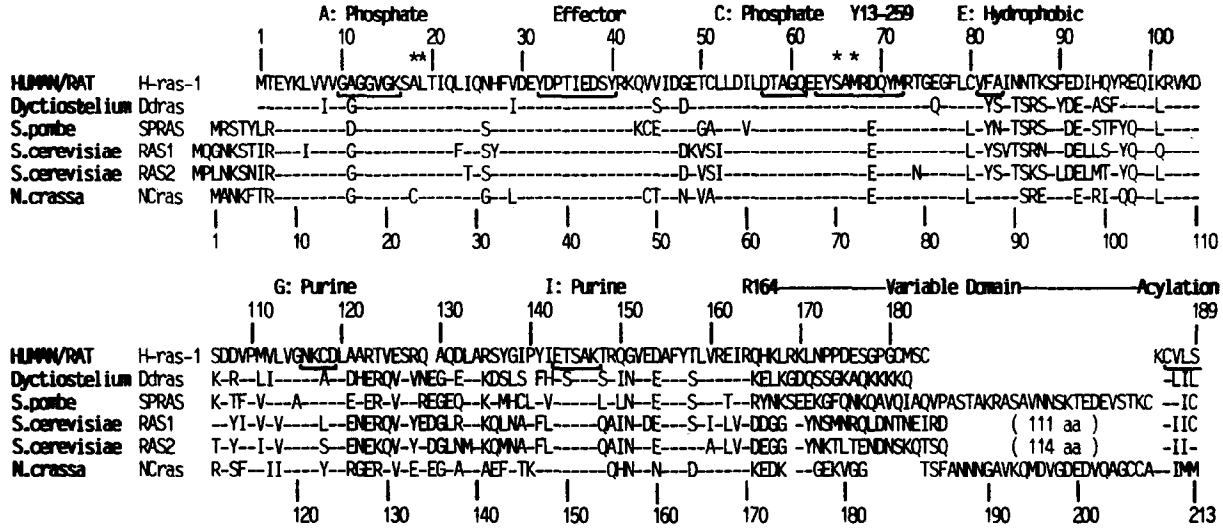

Fig. 3. Comparison of NC-ras amino acid sequence with those of human/rat *H-ras-1* and *ras*-protein sequences from lower eukaryotic organisms. Consensus sequences for the acylation motif, binding of GTP/GDP or of the Y13-259 neutralizing antibody, and the 'effector site' for interaction with GAP proteins, are indicted (underlined), as well as positions of some amino acids involved in oncogenic transformation (asterisks).

the alignment of the predicted amino acid sequences corresponding to human/rat *H-ras-1* proto-oncogenic polypeptide and the five known *ras* sequences from lower eukaryotic organisms, including *NC-ras*. The *Neurospora crassa ras* polypeptide is 84% homologous to the *H-ras-1* domain comprising the first 80 residues and 60% homologous to the next 84 amino acids. In this tract of 164 amino acids *NC-ras* shows the highest sequence identity with *H-ras-1*, compared with the reported lower eukaryotic *ras* sequences. The rest of the polypeptide, containing the so-called hypervariable domain, shows scarce homology, except for the CAAX acylation motif in the COOH terminal [2]. In this motif, the aliphatic amino acids (A) are isoleucine and methionine.

The *NC-ras* polypeptide contains all the four sequence elements GX_4GK^{S}/T (amino acids 15–22), DX_2G (amino acids 62–65), NKXD (amino acids 121–124) and EXSA (amino acids 149–152) involved in the interaction with GTP/GDP [43], the recognition of the Y13-259 neutralizing antibody, and the 'effector site' for interaction with GAP proteins [2,16,17]. All these sequences are 93% homologous to their equivalents of *H-ras-1*. For all these reasons it is evident that *NC-ras* is a new member of the *ras*-protein family.

Results reported in this paper show that *Neurospora crassa NC-ras* codes for a 24 kDa polypeptide that belongs to the *ras* family. This polypeptide contains the Gly^{17},Gly^{18}, Ala^{64} and Gln^{66} residues which are identical to the residues in positions 12, 13, 59 and 61 of normal *H-ras-1* protein in humans and mice. Changes in these positions lead to the abolition of *H-ras-1* GTPase activity in the presence of GAP. Taking in consideration all these results, together with the fact that *Saccharomyces cerevisiae RAS1* and *RAS2* code for proteins of about 41 kDa, it is evident that *Neurospora crassa NC-ras* and *Schizosaccharomyces pombe SPras* represent the lowest, conserved, evolutive antecedents of the *ras* p21–p24 family in a period of about 800 million years.

Acknowledgements: This study was partially supported by Fundación Antorchas-Argentina. D.L.A., A.M. and A.S. are Research Fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). F.B.A. is a Research Fellow of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (Argentina). H.N.T. is an Investigator of CONICET.

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