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Characterization of two breast tumor associated autoantigens, which are newly discovered gene products, has revealed that the first one (*Ngp-1*) is a GTP-binding protein which localizes exclusively to the nucleolus, and interacts with ribosomal protein L7. Possible interactions with other proteins are being investigated using the yeast two hybrid vector system. The *Ngp-1* gene is located on the short arm of human chromosome 1 at position 1p34-1p35. A clone containing the full length transcript of the second autoantigen clone *Auag2* has been obtained and is being sequenced to completion. Recombinant protein *Auag2* has been produced and purified for antiserum production. No part of the *Auag2* sequence is found in any of the databases, although one region shows some homology to a vascular endothelial growth factor. Additional breast cancer patient sera have been collected and will be tested for autoantibodies.

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FOREWORD

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Jarin Rackauckas 10/24/96

PI - Signature Date

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INTRODUCTION

Tumor growth is associated with the expression of mutated gene products, inappropriate gene expression, and the breakdown of tissue architecture, leading to the exposure and release into the peripheral circulation of sequestered antigens (1,2). Whether these circulating, mutated or newly displayed tumor-associated antigens elicit an autologous humoral immune response in the breast tumor patient is of vital interest. Isolation, identification and characterization of novel breast tumor associated autoantigens might yield new insights into the disease process, and moreover, may be developed into diagnostic screening tests and potential targets for immunotherapy.

The screening of cDNA expression libraries with autologous patient serum is a powerful technique, which has been used successfully for the identification of autoimmune disease antigens (3), and which we have adapted for the identification of autoantigens in cDNA libraries made from breast tumor mRNA. After screening cDNA libraries, derived from primary ductal breast carcinomas with autologous patient serum, we have detected and isolated two immunoreactive cDNA clones, both of which are newly discovered gene products. We propose to fully characterize the identified autoantigens and to construct additional cDNA libraries and screen them with autologous serum to identify and isolate additional breast tumor autoantigen cDNAs. The ultimate goals of our research project are: *1.* To isolate autoantigen clones which individually or in combination react specifically with most breast tumor patient sera and may form the basis for the development of diagnostic tests or perhaps identify potential targets for immunotherapy, and, *2.* To test the hypothesis that breast tumors result in the expression of a characteristic profile of autoantigens.

BODY

During the past year we have concentrated most of our research effort at characterizing the two autoantigenic gene products that we identified, both of which are newly discovered genes. The most progress has been made in the characterization of the first of our isolated breast tumor autoantigens, and the results of our initial studies have been published (4). In addition, a figure from our paper showing nucleolar staining with anti autoantigen antibody was chosen as the cover illustration for the february 1996 issue of Cell Growth and Differentiation (appendix, page 10).

We have named the gene encoding the first autoantigen *Ngp-1, (Genbank accession # L05425)*, and from the predicted amino acid sequence, have determined that it encodes a GTP-binding protein (5,6). Immunohistochemical analysis of tissue sections with affinity purified antiserum raised against a recombinant *Ngp-1* protein revealed that the antigen was exclusively localized in the nucleolus and nucleolar organizer regions in all cell types analyzed (hence our proposed name *Ngp-1:* Nucleolar G-Protein gene 1). The arrangement and spacing of the GTP binding protein motifs indicate that *Ngp-1* belongs to a newly described subfamily of GTPases with one other known human member (*HSR1*) (7), the others being of prokaryotic origin. The alignment of the GTP-binding protein motifs of *Ngp-1* with those of other members of the new subfamily are shown in the appendix, page 11. The discoverers of *HSR1* (Dr. Pontarotti et al, Centre National de la Recherche Scientifique, Toulouse, France) contacted me to request a full length probe for *Ngp-1*, and suggested a collaboration to determine the chromosomal location of the Ngp-1 gene. The results indicated that the Ngp-1 gene is located on the short arm of human chromosome 1, in the 1p35-1p34 region (appendix, page 12).

Southern blot analysis of *Eco R1* digested human genomic DNA and hybridization against a full length *Ngp-1* cDNA probe revealed two major bands hybridizing (approximately 15 and 8 kb in length). Two fainter bands were detected at 6 and 2.5 kb as well (appendix, page 13). Subcloning these genomic restriction fragments into standard vectors would be straight forward, however, sequencing this length of DNA would be a major timeconsuming undertaking which we will not begin at this time, since we feel there are more important projects to complete.

Since all GTPases interact with other cellular macromolecules (6), we set out to identify other gene products which interact with *Ngp-1* during its regulatory functions. To accomplish this we have subcloned the entire open reading frame portion of *Ngp-1* into

phagemid vector *pBD-GAL4* (the bait plasmid) which we have begun to test in the yeast two-hybrid vector system. The yeast two-hybrid vector system is one of the most efficient techniques available for detecting in-vivo protein interactions (8). In a request for anti *Ngp-1* antibody from a researcher in Germany (Dr. Stephan Witte, University of Konstanz) I learned that *Ngp-1* protein interacted strongly with human ribosomal protein L7. Human ribosomal protein L7 is itself an autoantigen, and is associated with Systemic Lupus Erythematosus (9). The interaction was observed by Dr. Witte using the yeast two hybrid vector system with human ribosomal protein L7 as the bait.

We are continuing our work on characterization of our second breast tumor autoantigen isolate (working name *Auag2*), which is also a newly discovered gene. No part of this gene is to be found in any of the gene sequence databases. This is surprising considering the recent proliferation of partial cDNA sequence data entered in the databases from entire cDNA libraries. A possible explanation for the absence of *Auag2* sequence data in the databases is that *Auag2* contains regions of extremely high GC content (appendix, page 14), probably making reverse transcription of the mRNA difficult because of secondary structure, hence *Auag2* is under-represented in cDNA libraries. The high GC content also makes accurate sequencing a difficult task, we have however deposited a partial sequence of *Auag2* in the Genbank database (*accession # U24576*). We were finally able to isolate a clone containing 2.1 kb of *Auag2* sequence (approximately the size of the Auag2 mRNA as determined by northern blot) by using the Gene Trapper technology (Life Sciences), where a cDNA library in a plasmid vector is converted to single strand form, then hybridized with gene specific biotinylated oligonucleotide probes which are captured by avidin coated magnetic beads, thus highly enriching for the desired gene product. In northern blots of mRNAs isolated from various human tissues, *Auag2* appears to be most highly expressed in testes and brain; and is not detectable in liver and kidney (appendix, page 15). In certain tissues such as skeletal muscle there appears to be an extra band; perhaps representing different splicing products. Recombinant *Auag2* protein has been produced and has been purified for antiserum production, which will be used for immunohistochemical localization of *Auag2* protein within different tissues and cell types.

In the last progress report we reported that we had isolated a third potential autoantigen, however this proved to be non-reproducible. In the interim we have collected additional serum samples from breast cancer patients, and plan to screen these for the presence of autoantibodies, reacting with specific autoantigens.

CONCLUSIONS

We have made considerable progress in characterizing autoantigen *Ngp-1*, which we have found to be a very unusual molecule because it is one of the few GTP-binding proteins with a nuclear localization. We have determined the chromosomal location of the *Ngp-1* gene, and learned that it interacts with ribosomal protein L7, itself a known autoantigen. Judging by the number of requests for probes and antisera there is great interest in this gene product. The second autoantibody clone *Auag2* has an unusually high GC content and is under-represented in cDNA libraries. We were able to isolate a full length clone using a hybridization affinity technique. Although it shows some homology to vascular endothelial growth factor in one region, it does not match up with anything presently in the databases.

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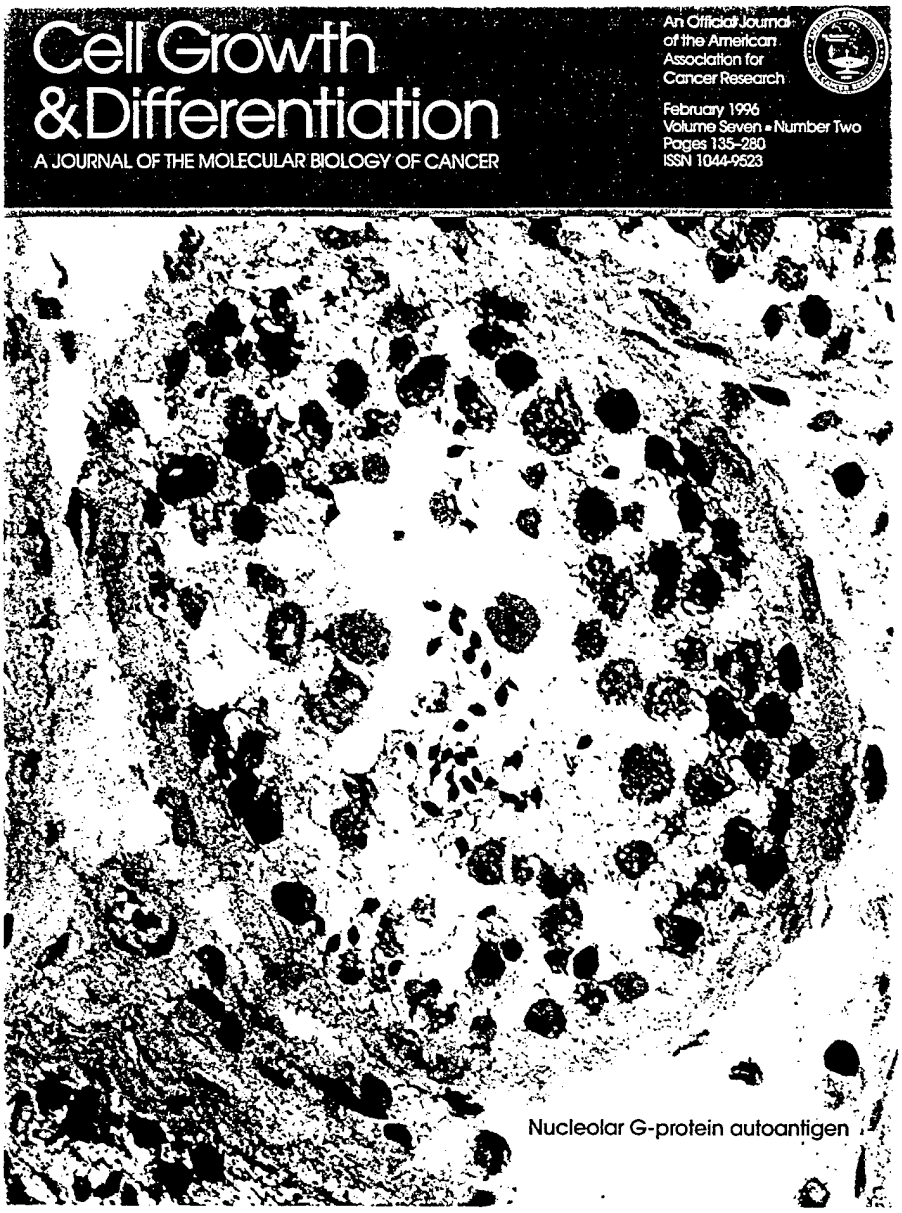


Cover illustration of February 1996 issue of Cell Growth and Differentiation, showing immunohistochemical stain of a human testis section, with antibody against *Ngp-1* (4). In the cross section of the seminiferous tubule shown, prominent staining is present in the nucleoli of cells nearer the basement membrane (spermatogonia, primary spermatocytes and Sertolli cells) as well as in interstitial Leydig cells.

C. Vernet et al.: New GTP-binding protein on MHC class I

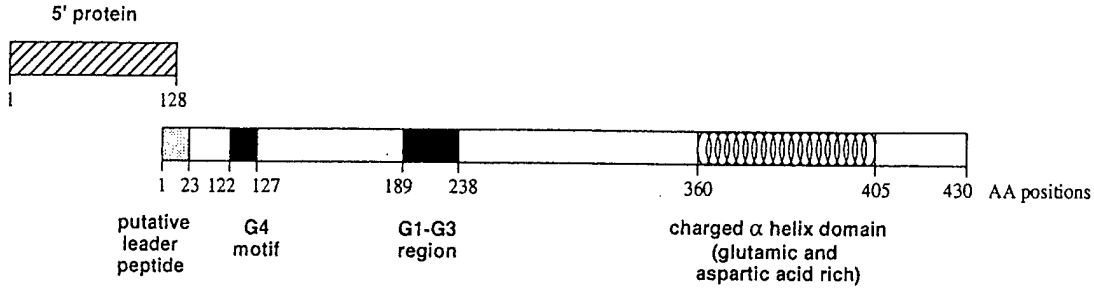


Fig. 1. Schematic representation of the whole protein HSR1. The 5′ ORF protein, overlapping the seven first amino acids of the HSR1 protein, is also presented.

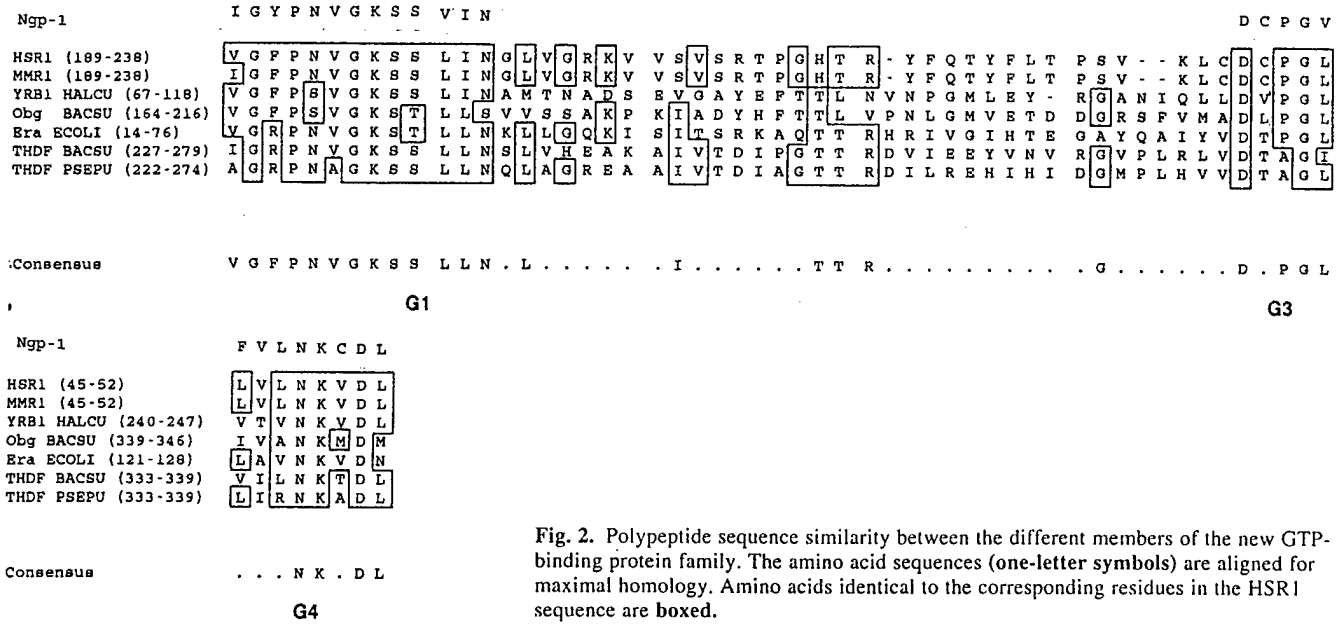
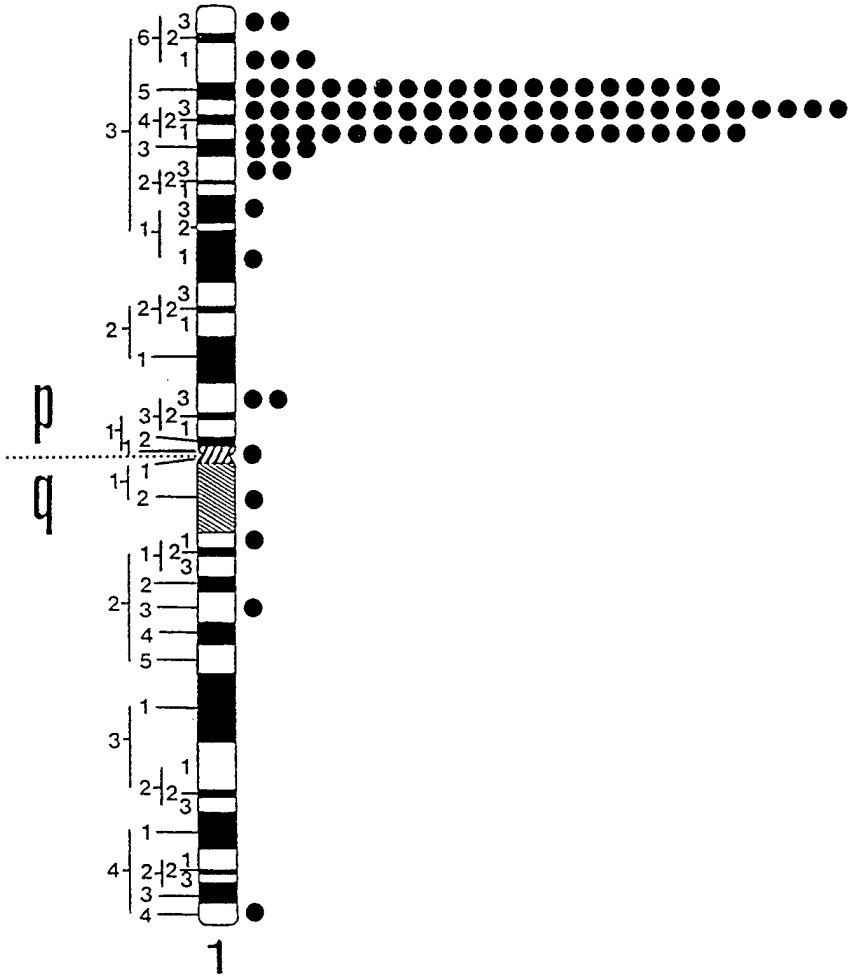
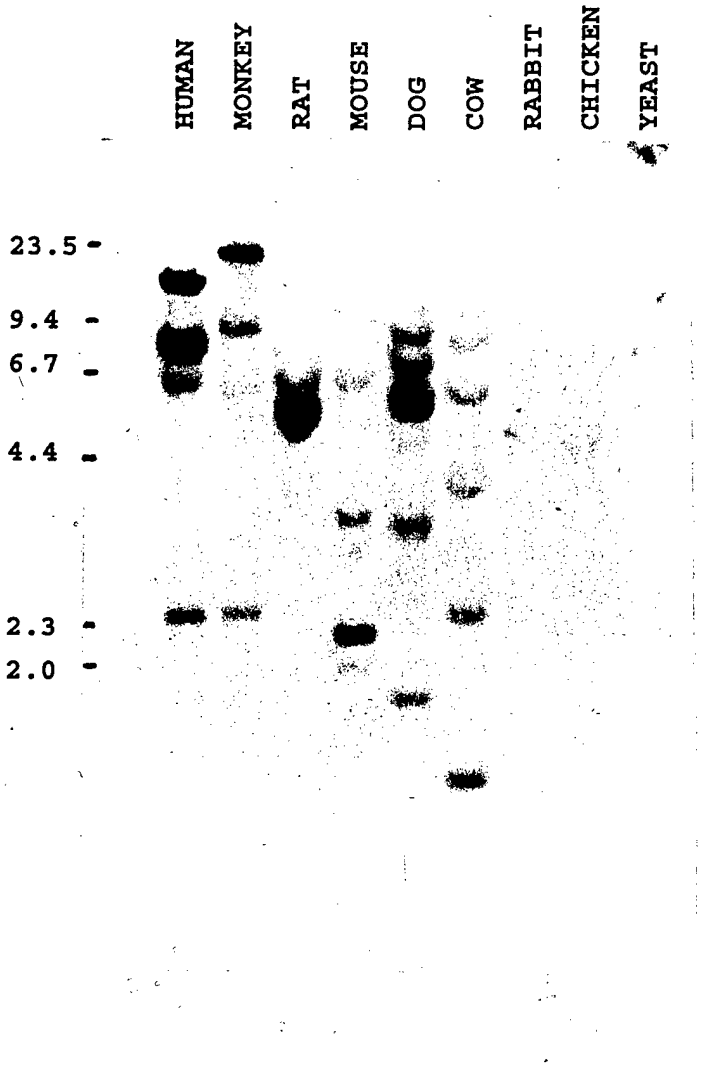


Fig. 2. Polypeptide sequence similarity between the different members of the new GTP-binding protein family. The amino acid sequences (**one-letter symbols**) are aligned for maximal homology. Amino acids identical to the corresponding residues in the HSR1 sequence are **boxed.**

Alignment of GTP-binding protein motifs of *Ngp-1* with those of other sub-family members. *Ngp-1* motifs are superimposed on figure from Vernet et al (7).



In-situ hybridization map of human chromosome 1, using a cDNA probe of *Ngp-1*, showing localization of signal to region 1p35-1p34.



Southern blot containing 4μg of EcoR I restriction digested genomic DNA per lane from nine eukaryotic species, hybridized against an *Ngp-1* probe. Species DNA in each lane is identified on top of blot, and position of λ/Hind III DNA size markers is noted on the left side of the blot.

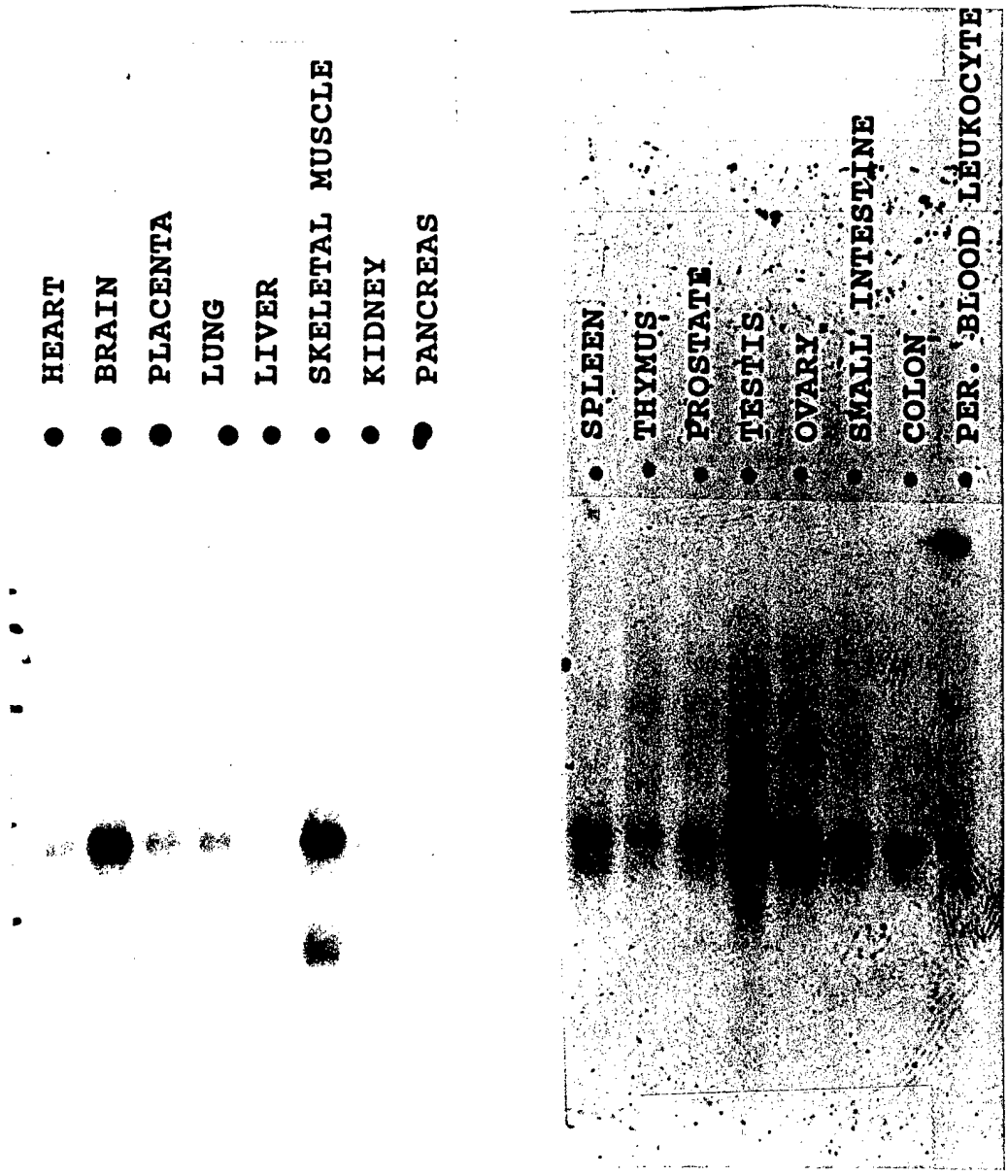
FILE NAME : AUTAG2.DNA

SEQUENCE : 587BP; 91 A; 214 C; 224 G; 58 T.

*** SEQUENCE LIST *** (SINGLE)

```
          10         20         30         40         50         60
5' TCGCGGAGGGAGCGAGCGAGCCGGCTAGAGGCCAGCTCCGCCGCCGCCGCCGCCTCCGAG
          70         80         90        100        110        120
   CCGGGCAGCAACAGTCCCCGGCAGGCGTGCGCAGGCTCCAGCGCCCCCGGCCCGGCCGGC
         130        140        150        160        170        180
   CGCAGCCCCCGACGCCTGGGTGCGCCTGCCTGCCGGCCTCCGCACCGTCGCCGCCGCTCC
         190        200        210        220        230        240
   CGCGGCCGCTGTTGTGTCTGCGACTGCTCCCGGCCGGAGGTGCAGGGAGCTCAGCCGAGC
         250        260        270        280        290        300
   CGCCGCTGCCAGCCCGGAGCGAGCAAGCGAGCGAGCCCGCAGGAGGAAGGAAGGCGGCGG
         310        320        330        340        350        360
   CGGAGGAGGAGGAGGAAGCGAGGACGCGGGCGGGGCGGGGGCGGCCGGGCGGGGAATATA
         370        380        390        400        410        420
   TCAAAGTGAAGCCACATTTGCCAAACTTGCAGCAGCGATTCGCAGCAGTTGCTGCCGCTG
         430        440        450        460        470        480
   CGGCCGCGCCTGAAGCCGCGCCGCGCGGCGCCGAGGGCTCCTGCAGCTGCGTCGCGCGGC
         490        500        510        520        530        540
   AGTCGGAGGCGGAGAAGGACGAAGACTGAGACTGACACTTCTGTCTCCCGGCCGCCCCGG
         550        560        570        580
   CACTTACGACGGGGGCCCCCCAACCCGCCCCAGAGCAACGGCGATTT 3'
```

Sequence of original autoantigenic *Auag2* isolate cDNA showing extremely high GC content (*Genbank accession # U24576*).



Northern blots containing approximately 2μg of poly A$^+$ RNA per lane from sixteen different human tissues (identified on top of blots) hybridized against an *Auag2* probe. Location of RNA size marker bands is indicated in the left margin of the blot; approximate size of major band is 2.1 kb.