Volume 274, number 1,2, 193-198 FEBS 09102 November 1990

Intracellular immunization

Cloning and intracellular expression of a monoclonal antibody to the p21^{ras} protein

Thomas M. Werge, Silvia Biocca and Antonino Cattaneo

CNR Institute of Neurobiology, Viale Carlo Marx 15, 00156 Roma, Italy

Received 1 August 1990; revised version received 26 September 1990

Following the demonstration that intracellular expression of antibodies ('intracellular immunization') may be utilized to engineer new traits in mammalian cells [1], we undertook experiments to perturb the function of p21^{ras} proteins, by engineering the intracellular expression of the anti-p21^{ras} antibody Y13-259 [2]. The variable regions of this antibody have been cloned and, after verifying their antigen binding activity, expressed in general purpose vectors for the intracellular expression of antibodies. The results confirmed that the cloned antibody has been efficiently expressed both in the secretory and the intracellular forms. Thus, intracellular immunization of mammalian cells against p21^{ras}, or any other antigen for which a monoclonal antibody is available, can now be performed.

Intracellular immunization; p21^{ras} protein; Antibody expression vector

1. INTRODUCTION

Ras genes encode for membrane associated GTP-binding proteins that are involved in the control of cell proliferation and differentiation [3]. Members of this gene family are highly conserved throughout evolution and are widely expressed in cells of both lower and higher eukaryotes. All the available evidence points to ras proteins as key regulatory molecules linking growth factor receptors to their signal transduction pathway(s). In *Saccharomyces cerevisiae* a genetic approach has led to the identification of functions for the RAS1 and RAS2 proteins as well as functions upstream and downstream of the RAS gene products [4]. In mammalian cells, where such an approach is not possible, the function of ras gene products remains unclear. However, microinjection of the anti-p21^{ras} antibody Y13-259 [2] was shown to block the serum stimulated DNA synthesis in NIH 3T3 cells, the nerve growth factor induced differentiation of PC12 cells, the transformation by a number of oncogenes as well as ras stimulated adenylate cyclase activity in yeast cells [3]. This antibody, therefore represents a valuable reagent to interfere with the function of p21^{ras}. However, the intrinsically transient nature of the antibody microinjection experiments limits the usefulness of such an approach to study ras gene functions in mammalian cells. We recently proposed the stable intracellular expression of monoclonal antibodies as a means to inactivate cellular proteins by demonstrating that heavy and light chains redirected to an intracellular compartment indeed associate to form functional antibodies [1]. We report in this paper the molecular cloning of the variable regions of the heavy and light chain of the monoclonal antibody Y13-259, and their reconstitution in general purpose vectors for extracellular and intracellular expression of immunoglobulins in mammalian cells. These constructs were transfected into cultured cells, confirming the subcellular localization of the expressed antibody chains. Experiments on intracellular immunization of cells against the p21^{ras} protein can therefore be performed.

Correspondence address: A. Cattaneo, CNR Institute of Neurobiology, Viale Carlo Marx 15, 00156 Roma, Italy

Abbreviations: PCR, polymerase chain reaction; NLS, nuclear localization sequence; FCS, fetal calf serum; BG, β-globin-promoter; V_H, variable region of the heavy chain; V_K, variable region of the light chain; $HuC_{\gamma 1}$, constant regions of the human heavy γ-1 chain; HuC_K, constant region of the human light κ chain

2. MATERIALS AND METHODS

2.1. *Plasmids and bacterial strains*

M13-V_HPCR1, M13-V_KPCR1, pSV-gpt-$Hu_{\gamma 1}$ and pSV-hyg-HuC_K were a gift from G. Winter (MRC Cambridge); plasmid p805 was received from M. Neuberger (MRC Cambridge) BW2029 bacteria (*E. coli* N6105 (Adhya Sankar, NIH) harbouring a temperature-sensitive λ-repressor controlling expression of p21 from pJCL-E30 [5]) were provided by B. Willumsen (University of Copenhagen).

2.2. *Cloning of immunoglobulin variable regions by PCR*

Total cytoplasmic RNA was prepared from Y13-259 hybridoma cells as descried [6]. First strand cDNA synthesis and PCR were performed as in [7], except that annealing was carried out at 60°C.

2.3. *Plasmid and mutant construction*

The β-globin promoter region was excised as a 850 bp *Hin*dIII/*Nco*I fragment from p805 and cloned into M13-V_HPCR1 and M13-V_KPCR1 [7], cut with *Hin*dIII/*Nco*I, giving M13-βG-V_HPCR and M13-βG-V_KPCR. The PCR amplified DNA was force cloned into

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

A **Sequence of Y13-259 V_H**

```
1                                                   10                                                  20
 Q   V   Q   L   Q   Q   S   G   G   G   L   V   Q   P   G   R   S   L   K   L
CAG GTC CAA CTG CAG CAG TCT GGA GGA GGC TTA GTG CAG CCT GGA AGG TCC CTG AAA CTC
          PstI
21                                                  30          CDR1                                    40
 S   C   V   V   S   G   F   T   F   S   N   Y   G   M   N   W   I   R   Q   T
TCC TGT GTA GTC TCT GGA TTC ACT TTC AGT AAC TAT GGA ATG AAC TGG ATT CGC CAG ACT

                                                    50      52  A               CDR2                .
 P   G   K   G   L   E   W   V   A   Y   I   S   S   G   S   S   Y   L   Y   Y.
CCA GGG AAG GGA CTG GAG TGG GTT GCA TAC ATT AGT AGT GGT AGC AGT TAC CTC TAC TAT

60                                                  70
 A   E   T   V   K   G   R   F   T   I   S   R   D   N   A   K   N   T   L   Y
GCA GAA ACG GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC

80      82  A   B   C                                   90                              .
 L   Q   M   T   S   L   R   S   E   D   T   A   L   Y   Y   C   A   R   H   E.
CTG CAA ATG ACC AGT CTG AGG TCT GAA GAC ACT GCC TTG TAT TAC TGT GCA AGA CAT GAG

    CDR3    100 A   B   C           .
 G   T   G   T   D   F   F   D   Y   W   G   Q   G   T   T   V   T   V   S   S
GGT ACG GGT ACC GAC TTC TTT GAT TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
                                                                    Bst E
```

B **Sequence of Y13-259 V_K**

```
1                                                   10                                                  20
 D   I   Q   L   T   Q   S   P   H   S   L   S   A   S   L   G   E   T   V   S
GAC ATC CAG CTG ACC CAG TCT CCA CAT TCC CTG TCT GCA TCT CTG GGA GAA ACT GTC TCC
        PvuII
                        CDR1        30                                                              40
 I   E   C   L   A   S   E   G   I   S   N   Y   L   A   W   Y   Q   Q   K   P
ATC GAA TGT CTA GCA AGT GAG GGC ATT TCC AAT TAT TTA GCG TGG TAT CAG CAG AAG CCA

                                                    50          CDR2                                    60
 G   K   S   P   Q   L   L   I   Y   Y   A   S   S   L   Q   D   G   V   P   S
GGG AAA TCT CCT CAG CTC CTG ATC TAT TAT GCA AGT AGC TTG CAA GAT GGG GTC CCA TCA

                                                    70                                                  80
 R   F   S   G   S   G   S   G   T   Q   F   S   L   K   I   S   N   M   Q   P
CGG TTC AGT GGC AGT GGA TCT GGC ACA CAG TTT TCT CTC AAG ATC AGC AAC ATG CAA CCT

                                                    90              CDR3                                100
 E   D   E   G   V   Y   Y   C   Q   Q   A   Y   K   Y   P   S   T   F   G   A
GAA GAT GAA GGG GTT TAT TAC TGT CAA CAG GCT TAC AAG TAT CCT TCC ACG TTT GGA GCT

 G   T   K   L   E   I   K
GGG ACC AAG CTG GAG ATC AAA
          <BglII/BclI>
```

Fig. 1. Nucleotide and amino acid sequence of Y13-259 heavy (A) and light (B) variable regions. The PCR primers and the adjacent flanking regions are underlined.

these M13 vectors [7], giving M13-βG-V_H259 and M13-βG-V_K259 (Fig. 4), and subsequently sequenced [8]. The *Hin*dIII/*Bam*HI fragments from these vectors were recloned in the modified pSV-gpt and pSV-hyg vectors [7], giving pβG-V_H259 and pβG-V_K259.

The secretory leader sequence from the mouse V47 V_H gene [9] utilized in the M13-PCR vectors was substituted by the nuclear localization sequence (NLS) as described [3] giving the M13-βG/NLS-V_H259 and M13-βG/NLS-V_K259 (Fig. 4). *Hin*dIII/*Bam*HI fragments from these plasmids were then inserted into the pSV-gpt and pSV-hyg expression vectors, giving pβG/NLS-V_H259 and pβG/NLS-V_K259.

2.4. *Cells, transfections, immunofluorescence and Western blot*

Rat pituitary GH3 [10] and simian COS cells were cultured in DMEM with 10% FCS and transfected by DEAE-dextrane/chloroquine [11]. Indirect immunofluorescence was performed as described in [1]. Expression of the $p21^{ras}$ protein in BW2029 exponentially growing at 30°C was induced overnight at 42°C and harvested as described [5]. The $p21^{ras}$ protein was revealed by Western blotting [5].

3. RESULTS

The variable regions of the heavy and light chain of the rat monoclonal antibody Y13-259 [2] were cloned by the polymerase chain reaction (PCR) method, as recently described [7]. The sequences were confirmed on different clones obtained from independent amplification reactions. Fig. 1 shows the nucleotide sequence of the heavy and light chain variable regions, between the two amplification primers (underlined), together with the deduced amino acid sequence. The amino acid sequences corresponding to the regions of the PCR primers are likely to differ in Y13-259. The complementarity determining regions (CDR) are overlined. Inspection of the Kabat data base showed that the sequences obtained are different from all other immunoglobulin variable regions.

The V_H and V_K gene regions of Y13-259 antibody were assembled together with human $C\gamma$ and C_K genes respectively, in vectors for expression of secreted rat/human chimaeric antibodies, pβG-V_H259 and pβG-V_K259. Cotransfection of these constructs into the cell lines COS and GH3 gave rise to high expression of the corresponding antibody chains. Fig. 2 shows corresponding fields of COS cell transfectants stained by double indirect immunofluorescence for light (Fig. 2A) and heavy (Fig. 2B) chain. Both double positive and single positive cells are present in the cell population. The staining pattern is typical of a secretory protein.

The supernatant of the COS double transfectants was assayed by Western blotting for the production of anti-$p21^{ras}$ antibodies (Fig. 3). The experiment demonstrates that these cells indeed secrete, albeit in low amounts, antibodies that recognize $p21^{ras}$ protein (Fig. 3, lane 3) as do the parental Y13-259 antibodies (Fig. 3, lane 1).

The secretory form of the cloned Y13-259 antibody is unlikely to interact with its corresponding antigen $p21^{ras}$ inside the cell, due to their different intracellular localization. In order to redirect the antibody away from the secretory pathway, the hydrophobic core of the secretory leader was substituted with the nuclear localization signal PKKKRKV from the SV40 large T

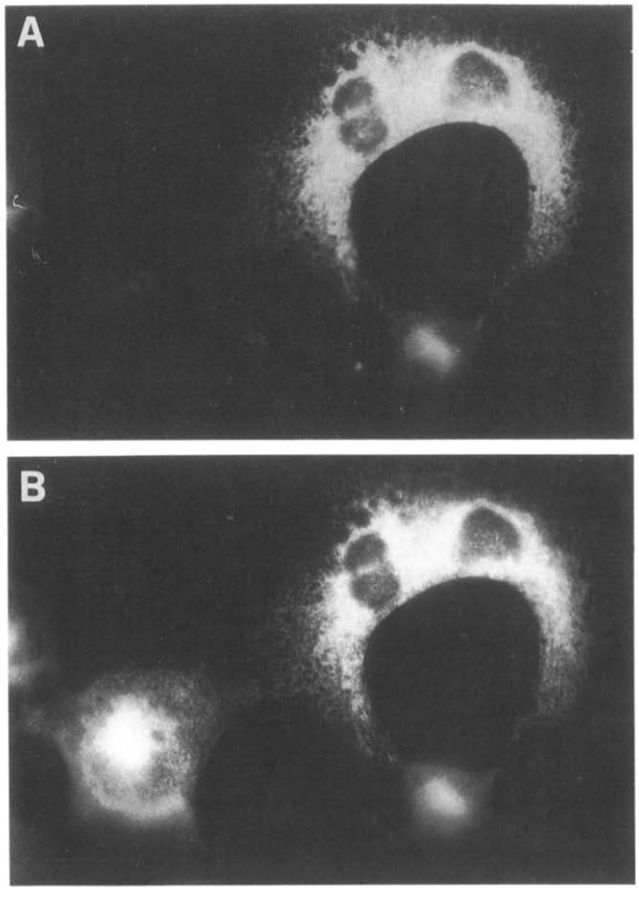

Fig. 2. Coexpression of secretory heavy and light chains from Y13-259 in COS cells transfected with pbG-V_H259 and pbG-V_K259. 48 h after transfection cells were stained by double indirect immunofluorescence with anti-light chain (A) and anti-heavy chain (B) antibodies. The same field is shown for the two fluorochromes used.

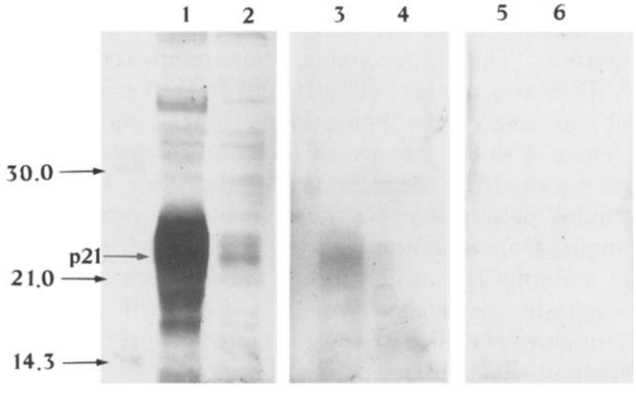

Fig. 3. Western blot analysis of COS cell transfectants. Cell extracts from heat shock induced (lanes 1, 3, 5) or uninduced (lanes 2, 4, 6) BW2029 bacteria were probed after SDS-PAGE with Y13-259 antibodies (lanes 1, 2) and with supernatant from COS cells transiently transfected with pβG-V_H259 and pβG-V_K259 (lanes 3, 4) or with corresponding plasmids encoding a non-relevant antibody (lanes 5, 6). The molecular size markers are indicated with arrows as well as the position of bacterial produced p21; M_w approx. 23 kDa.

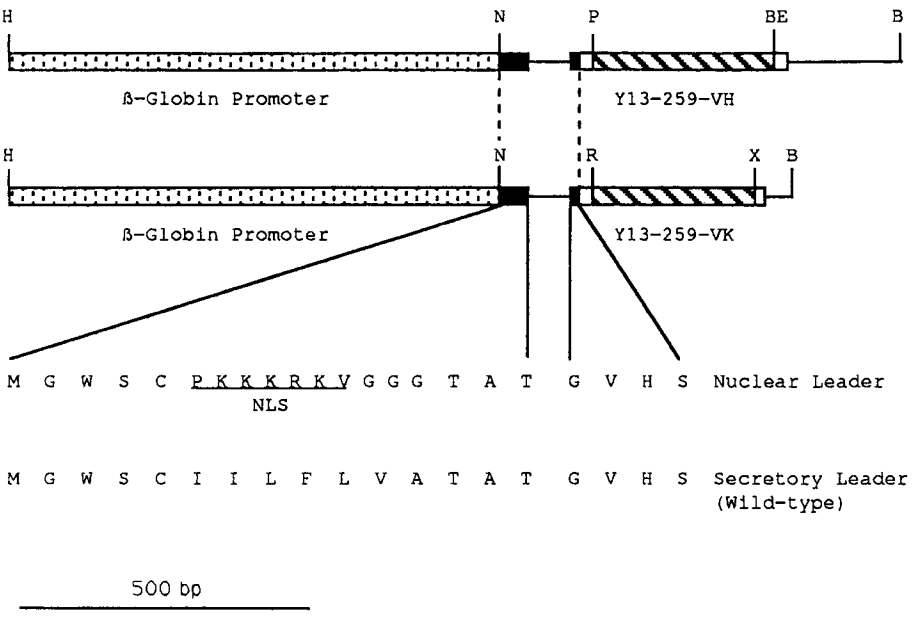

Fig. 4. Shuttle vectors for expression of immunoglobulin genes. The amino acid sequences of the wild-type secretory leader and of the nuclear localization leader are shown. The Y13-259 sequences (hatched boxes) and the adjacent flanking regions (unfilled boxes) correspond to the sequences shown in Fig. 1. The lines represent the leader intron and part of the V-C intron. Any variable region amplified with the primers described in [11] can be cloned into these vectors. H = *Hin*dIII; N = *Nco*I; P = *Pst*I; BE = *Bst*EII; B = *Bam*HI; R = *Pvu*II; X = *Bcl*I/*Bgl*II (the *Bcl*I site in the parental vector is lost upon cloning).

antigen [12] (Fig. 4), which we have shown directs immunoglobulin chains away from the secretory pathway and to the cytoplasm, from where, in some cell types, they reach the nucleus [1]. The NLS was incorporated into M13-βG-V_H259 and M13-βG-V_K259 (Fig. 4). After further cloning the activity of the final 'intracellular' expression vectors, pβG/NLS-V_H259 and pβG/NSL-V_K259, was determined by immunofluorescence after transient transfection into COS cells. Results for the heavy chain transfectants are shown in Fig. 5. Many cells in the transfected population show a bright signal for this antibody chain, demonstrating that this polypeptide chain are correctly and efficiently synthesized. The intracellular staining pattern (Fig. 5C,D,E) was remarkably different from that obtained for the secretory antibody chains (Figs. 2 and 5A), in keeping with the engineered direction of the antibody chain away from the secretory compartment. Different staining patterns can be found in the cell population, ranging from a predominant nuclear staining (Fig. 5C) to stainings in which both the nucleus and the cytoplasm are labelled (Fig. 5D). Cells with only the cytoplasm labelled are also found (Fig. 5E). Qualitatively similar results are obtained for the light chain (not shown), although the intensity of the signal is lower.

4. DISCUSSION

We have recently demonstrated the feasibility of expressing monoclonal antibodies in different intracellular compartments to interfere with the function of selected intracellular antigens [1], as one possible strategy to achieve 'intracellular immunization' [13] in mammalian cells. As a first application of this experimental strategy, we undertook experiments to perturb the function of the protooncogene c-ras. The monoclonal antibody Y13-259 is able to neutralize c- and v-ras activity following microinjection into living cells. We have therefore cloned the variable regions of this antibody and confirmed the antigen binding activity of the reconstituted secretory antibody by assaying the supernatants of transiently transfected COS cells. The cloned antibody does indeed recognize specifically $p21^{ras}$ protein, as the parental antibody (Fig. 3). The levels of antibodies secreted by COS cell transfectants appear to be quite low, possibly due to (i) the small percentage of the cells in the transfected population expressing the antibodies, and (ii) the reported low efficiency of antibody secretion by COS cells and other fibroblast cell lines [14]. We are presently deriving stable cell lines (NSO myeloma and GH3 pituitary cells) secreting the recombinant Y13-259 antibody (work in progress). We then engineered general purpose vectors for the intracellular expression of any antibody of interest. The results obtained confirm that the cloned antibody has been successfully expressed both in the secretory and the intracellular versions. It is noteworthy that the levels of expression of the intracellular heavy chain are comparable to its secretory counterpart (Fig. 5). We further show that the nuclear localization sequence (NLS) has proven efficient also for the heavy

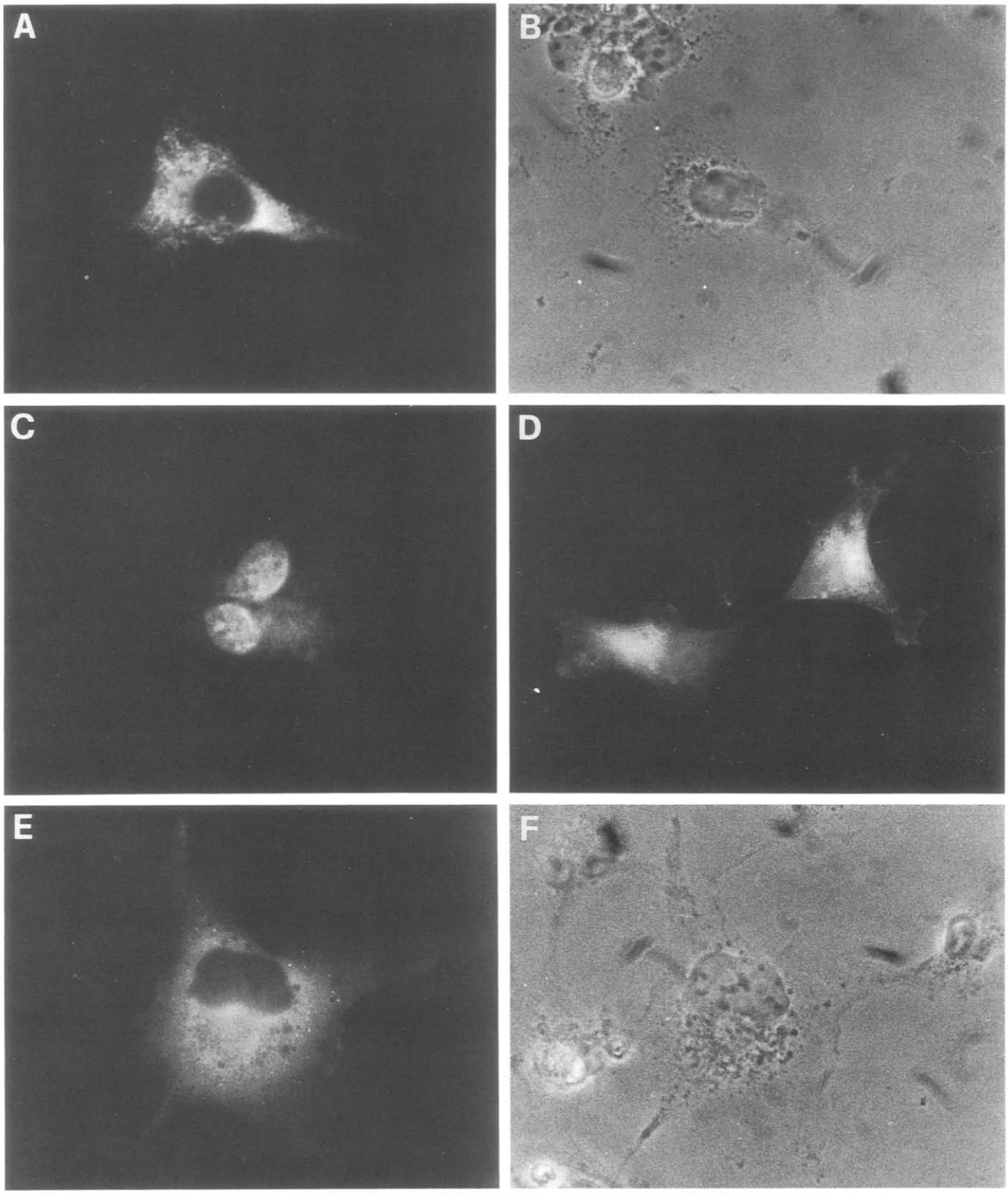

Fig. 5. Cellular distribution of the secretory (A) and intracellular (C, D and E) Y13-259 recombinant heavy chain. COS cells were transfected with pβG-V_H259 and pβG/NLS-V_H259 and stained 48 h later with anti-heavy chain antibodies. B and F show phase contrast pictures of the fields in A and E, respectively.

chain, as previously reported for the light chain [1], both in terms of preventing secretion, expression levels and targeting to the nucleus (Fig. 5). The fluorescence signal obtained for the light chain is somewhat lower, possibly due to cleavage of the constant domain or masking by some intracellular protein. This is consistent with our previous finding [1] that probing for intracellular antibodies with anti-idiotypic antibodies gives a higher proportion of nuclear labelling than probing with anti-isotype antibodies. The presence of the NLS targeting signal on the anti-$p21^{ras}$ antibody might divert the $p21^{ras}$ protein from its normal localization, thus contributing to inhibit its function.

The results presented show that the general purpose vectors engineered for intracellular expression of antibodies perform very efficiently and can therefore be utilized to study the phenotype resulting from the expression of any antibody of interest, in different cell types. In particular, it is now possible to derive cell lines in which the function of the $p21^{ras}$ protein is inhibited, thereby allowing the study of its role(s) in different physiological and pathological contexts.

Acknowledgements: We thank A. Bradbury and P. Piccioli for helpful discussions and suggestions, G. Winter, M. Neuberger and B. Willumsen for the kind gifts of DNA and bacterial strains, and A. Di Luzio for excellent assistance. We are grateful to P. Calissano for constant support and laboratory space. The work was supported through grants from the Associazione Italiana per la Ricerca sul Cancro, CNR (Prog. Fin. Biotecnologia e Biostrumentazione), The Preuss Foundation to A.C. T.M.W. acknowledges fellowships from Sigma-Tau and Grosserer L.F. Fogth Foundation, Copenhagen. S.B. is on leave of absence from the Dipartimento di Scienze Biochimiche, Universitá 'La Sapienza', Roma. A.C. is also at the Dipartimento di Fisiologia Generale, Universitá di Napoli.

REFERENCES

[1] Biocca, S., Neuberger, M.S. and Cattaneo, A. (1990) EMBO J. 9, 101-108.
[2] Furth, M.E., Davis, L.J., Fleurdelys, B. and Scolnick, E.M. (1982) J. Virol. 43, 294-304.
[3] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779-828.
[4] Hall, A. (1990) Cell 61, 921-923.
[5] Lacal, J.C., Santos, E., Notario, V., Barbacid, M., Yamazaki, S., Hsiang-Fu Kung, Seamans, C., McAndrew, S. and Crowl, R. (1984) Proc. Natl. Acad. Sci. USA 81, 5305-5309.
[6] Bradbury, A., Belt, K.T., Neri, T.M., Milstein, C. and Calabi, F. (1988) EMBO J. 7, 3081-3086.
[7] Orlandi, R., Gussow, D.H., Jones, P.T. and Winter, G. (1989) Proc. Natl. Acad. Sci. USA 86, 3833-3837.
[8] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
[9] Neuberger, M.S. (1983) EMBO J. 2, 1373-1378.
[10] Bancroft, F.C. (1981) in: Functionally Differentiated Cell Lines (Sato, G. ed.) pp. 47-59, Alan R. Liss, New York.
[11] Pelham, H.R.B. (1984) EMBO J. 3, 3095-3100.
[12] Kalderon, D., Roberts, B.L., Richardson, W. and Smith, A. (1984) Cell 39, 499-509.
[13] Baltimore, D. (1988) Nature 335, 395-396.
[14] Cattaneo, A. and Neuberger, M.S. (1987) EMBO J. 6, 2753-2758.