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Expression and targeting of intracellular antibodies in mammalian cells

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Genes encoding the heavy and light chains of a hapten-specific IgM antibody were modified by site-directed mutagenesis to destroy the hydrophobic leader sequences and allow expression in the cytoplasm of non-lymphoid cells. The *in situ* assembly of the mutant heavy and light chains was tested in transfected cell lines by immunofluorescence using anti-idiotypic antibodies. A positive diffuse cytoplasmic staining was observed. This demonstrated that the antibody polypeptide chains could assemble in the cell cytoplasm and led us to ask whether antibodies could be further targeted to the nucleus. Mutations were therefore made in which the leader sequence of the light chain was replaced by the nuclear localization signal of the SV40 large T antigen. Transfectants in which the heavy chain lacking the hydrophobic leader was expressed together with a light chain carrying the nuclear localization signal were selected and a nuclear distribution of the assembled antibody was found. Thus, it should prove possible to target a specific antibody to the cell nucleus with the aim of interfering with the function of a nuclear antigen.

Key words: intracellular antibodies/nuclear localization signal/targeting

Introduction

The secretion of immunoglobulins by transfectants of non-lymphoid cells has recently been demonstrated (Cattaneo and Neuberger, 1987; Weidle *et al.*, 1987). Such studies have allowed one to test the feasibility of utilizing the expression of specific monoclonal antibodies in selected non-lymphoid cells to interfere with the function of gene products active in the extracellular environment of an otherwise intact tissue, for example the nervous system (Cattaneo, 1988).

The approach of utilizing the stable expression of monoclonal antibodies by mammalian non-lymphoid cells to inactivate specific gene products could, in principle, be further refined by engineering the expression of antibodies in the cell cytoplasm. Although previous experiments (Cattaneo and Neuberger, 1987) have shown that assembly of functional antibodies will occur with comparable efficiency in the secretory compartments of both lymphoid and non-lymphoid cells, it has yet to be proven that such an assembly can occur at all in the reducing environment of the mammalian cell cytoplasm. Here we show that not only can antibody heavy and light chains be synthesized and assembled in the cytoplasm, but we further show that the assembled molecule can also be targeted to the nucleus.

Results

The mutated immunoglobulin genes

The DNA sequences encoding an IgM antibody with specificity for the hapten 4-hydroxy-3-nitrophenacetyl (NP) were modified by site-directed mutagenesis to direct the corresponding antibody polypeptide chains to the cell cytoplasm rather than to the secretory compartment. To this end, the hydrophobic amino acid core of the signal peptide for secretion was substituted with hydrophilic residues; the resultant mutant heavy and light chains are designated μ* and λ*. Mutations were made on the plasmids pSV-CMVλ1 and pSV-HSμ1. These plasmids direct the synthesis of secretory immunoglobulin light chain (under the control of the promoter for the human cytomegalovirus early gene) and heavy chain (under the control of the promoter of the *Drosophila hsp70* heat-shock gene) respectively. Both sets of constructs utilize the same leader sequence for secretion, namely that of the mouse V47 unrearranged V_H gene (Neuberger, 1983), so that the same mutagenic oligonucleotide was used to mutate the leader of both heavy and light chains. The hydrophobic amino acid core was replaced by an equal number of hydrophilic amino acids (cytoplasmic leader; Figure 1A and B). This hydrophilic signal sequence has previously been shown to target heavy chain mRNA to free (as opposed to membrane bound) polysomes, where it is translated into heavy chain polypeptides which do not become glycosylated (Sitia *et al.*, 1987; Mason *et al.*, 1988).

In order to investigate whether it might be possible to target cytoplasmic antibodies to other intracellular compartments the plasmid pSV-CMVλ^N (Figure 1B) was constructed. We took advantage of the nuclear localization signal PKKKRKV of the large T antigen of SV40 virus (Kalderon *et al.*, 1984). This sequence has been shown to act as an autonomous targeting signal able to target cyoplasmic proteins to the nucleus. By substituting the hydrophobic core of the V-region leader sequence by this signal, a polypeptide is obtained which should not be secreted but rather should be synthesized in the cytoplasm from where it should be translocated to the nucleus (λ^N). Three glycine residues were introduced downstream of the nuclear localization signal as a spacer, to ensure exposure of the nuclear leader from the folded molecule.

Immunolocalization of the mutated antibody chains

The activity of the plasmids described above was determined by transfecting them individually into COS cells and transiently assaying the cells by immunofluorescence in order to study the intracellular distribution of the antibody chains encoded for by the different plasmids (Figure 2). In particular, the staining observed with anti-light chain

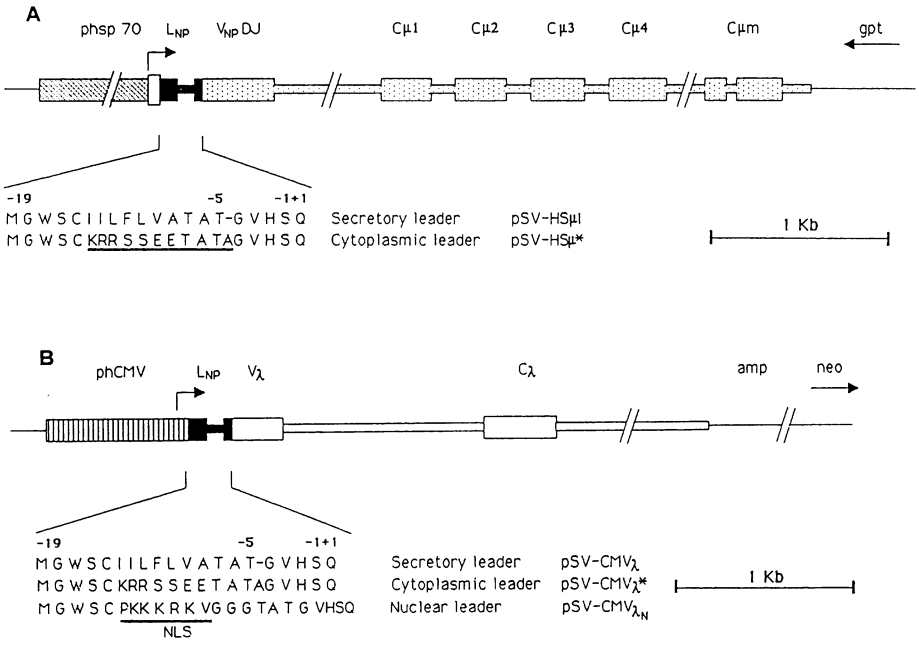

Fig. 1. Structure of plasmids. (**A**) Structure of pSV-HSμl and pSV-HSμ*. The thin line denotes the pSV2-*gpt* vector, the hatched box the *Drosophila hsp70* promoter region (phsp 70), the filled box the leader exon (L_{NP}), the open box a 60 nt stretch of the 5′ untranslated region of tk and the dotted box the μ heavy chain variable and constant region. The hydrophobic core of the wild-type secretory leader has been mutated as shown (cytoplasmic leader). (**B**) Structure of the pSV-CMVλ1, pSV-CMVλ* and pSV-CMVλ^N. The thin line denotes the pSV2-*neo* vector, the hatched box the human cytomegalovirus promoter region, the filled box the leader exon derived from the heavy chain variable region (L_{NP}) and the open box the λ variable and constant region. The hydrophobic core of the secretory leader has been mutated into a 'cytoplasmic leader' and 'nuclear leader' as shown.

antibodies in cells transfected with λ* plasmid (Figure 2A) is diffuse and not associated to any particular cellular structure, as expected for a cytoplasmic polypeptide.

The staining of cells transfected with the λ^N plasmid (Figure 2B) is very clearly confined to the nuclei, showing that the nuclear localization sequence of the large T antigen of SV40 positioned at the N terminus of the light chain is correctly recognized.

The cytoplasmic heavy chain μ*, which carries the same mutated leader sequence as λ*, is also diffusely distributed (Figure 2C). In this case, however, a strong fluorescent signal is also seen in a structure which is induced in the cells by the heat shock treatment necessary to express the μ* polypeptide (see also the interference contrast to the same group of cells, Figure 2G). In fact, a similar staining was seen when cells expressing the λ* polypeptide were heat-shocked (not shown). The intracellular distribution of the μ* polypeptide is clearly different from that of a secretory μ heavy chain, as shown in Figure 2D (COS cells transfected with the plasmid pSV-CMVμ, which encodes for a μ heavy chain under the control of human cytomegalovirus promoter).

Expression of heavy and light immunoglobulin chains in the cytosol

Plasmids pSV-CMVλ* and pSV-HSμ* (which contain the selectable markers *neo* and *gpt* respectively) were then stably transfected into a number of cell lines (Table I) and immunoprecipitation studies were performed. Comparison of the secretory and cytoplasmic μ heavy chain in the transfected cells (Figure 3A, lanes 1 and 2) on a low percentage SDS−PAGE gel revealed a difference in their apparent mol. wts; this is consistent with the absence of glycosylation of the cytoplasmic μ* chains. The low mol. wt bands present in the cytoplasmic heavy chain immunoprecipitate (lane 2) are most probably due to degradation products; this reflects the rapid turnover of this protein as demonstrated by Sitia *et al.* (1987). The cytoplasmic λ* polypeptide was immunoprecipitated from C6λ*μ* cells (C6 glioma cells transfected with the pSV-CMVλ* and pSV-HSμ* plasmids) using anti-λ antibodies (Figure 3B, lanes 1, 2 and 3). A band corresponding to the light chain is precipitated from the cytoplasmic transfectants, although it is less abundant than in the corresponding secretory cell line C6λμ (Figure 3B, lane 4). The small difference in mol. wt between the two light chains is expected, since the secretory λ chain is not glycosylated and the mutated signal peptide (~1 kd) should be retained in the cytoplasmic chain. As expected, in C6λμ cells, antibodies against the light chain also immunoprecipitate the assembled μ heavy chain (Figure 3, lane 4). Interestingly, such coprecipitation is also seen in all the C6λ*μ* transfectant clones tested indicating that there is assembly of the cytoplasmic heavy and light chains (Figure 3B, lanes 1, 2 and 3). The excess of light over heavy chains visible in the immunoprecipitates from C6λ*μ* cells indicates that ~40% of the μ polypeptide synthesized in the cytosol is found to be associated to light chain, as confirmed by subsequent precipitation with anti-μ antibodies (not shown). However, the levels of cytoplasmic heavy and light chain expressed by these cells are much lower than those of their secretory counterparts, in keeping with the rapid turnover of the cytoplasmic heavy chain (Sitia *et al.*, 1987). Possible reasons for this rapid turnover are discussed below (see Discussion). Addition of antigen (NP bound to bovine serum albumin) to the extraction buffer results in the recovery of a higher proportion of assembled intracellular antibodies (not shown) suggesting that the antigen can help the associaiton of heavy and light chains.

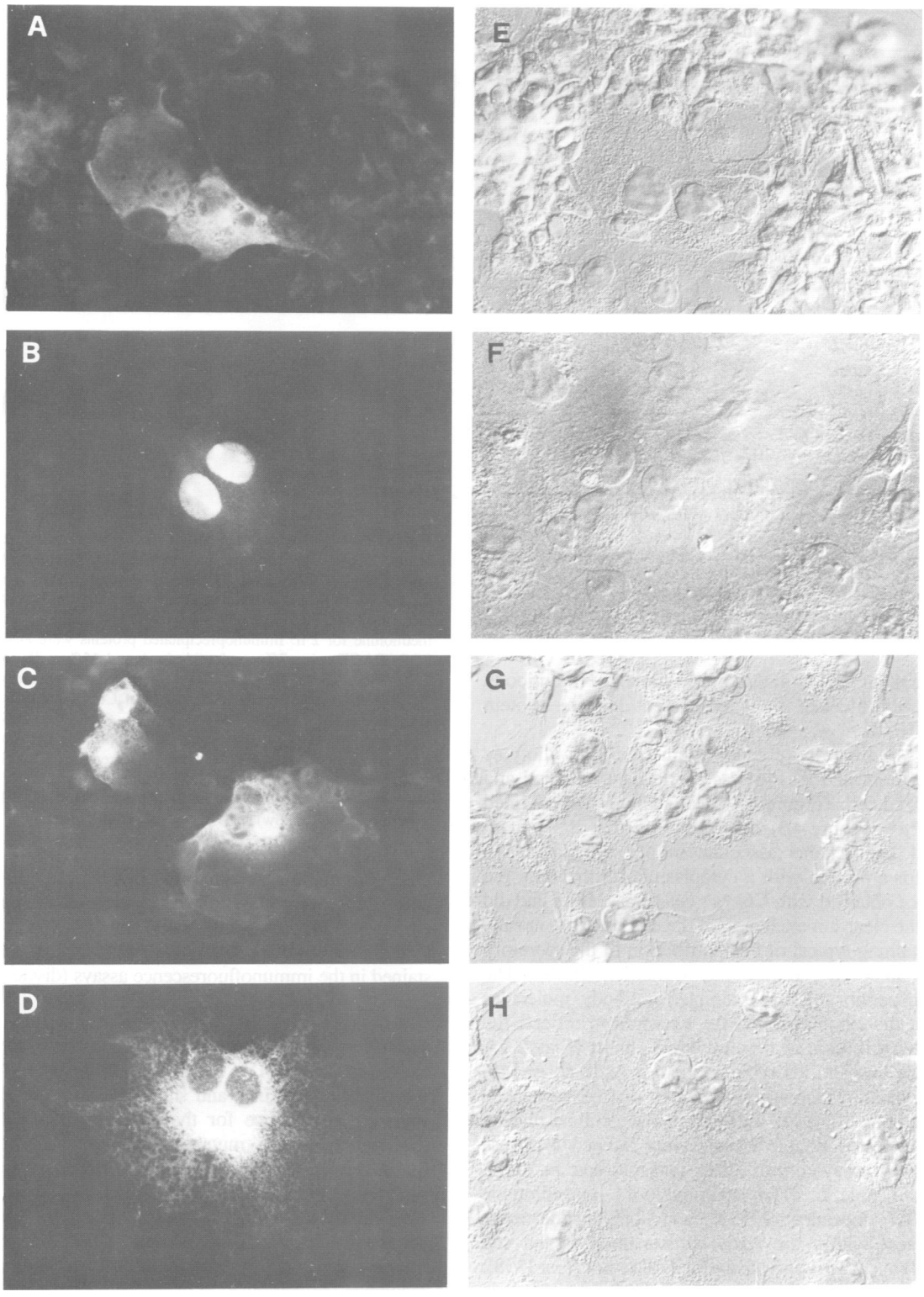

Fig. 2. Immunofluorescence of transiently transfected COS cells. (**A** and **B**) COSλ* and COSλ^N stained with anti-λ antibodies. (**C** and **D**) COSμ* and COSμ stained with anti-μ antibodies. (**E, F, G** and **H**) are interference contrast views of the corresponding fields as in A, B, C and D respectively.

In situ assembly of antibody chains

The experiments reported in the previous section suggest that the 'cytoplasmic' heavy and light chains do indeed associate *in vivo*, but do not formally prove this, since the possibility of an association during extraction cannot be ruled out. However, in order to demonstrate that this was unlikely we used two different monoclonal antibodies (Ac38 and Ac146; Reth *et al.*, 1979) directed against the idiotype of the anti-NP antibody to show that cytoplasmic association was occurring *in situ*. These antibodies recognize two different idiotypes formed by the association of the heavy and light chain variable regions, but not by either on its own. Binding

Table I. Intracellular antibodies in different cell lines

Parental cell line[a]	Plasmid transfected	Fluorescence anti-light chain[b]	Fluorescence anti-idiotype[b]
A			
J558L	μ^*	++++[c]	−
J558L	μ^*/λ^*	++++[c]	+
C6	μ^*/λ^*	+	+
Ltk$^-$	λ^*	+	−
COS	μ^*/λ^*	++	++
B			
P3.X63.Ag8	μ^*/λ^N	++	++(C)[d]
Ltk$^-$	λ^N	++	−
C6	μ^*/λ^N	++	++(C)
CS	μ^*/λ^N	++	++(C/N)
COS	μ^*/λ^N	+++	+++(C/N)

[a]Cell lines were tranfected as described in Materials and methods with the following plasmids: μ^*, pSV-HSμ^*; λ^*, pSV-CMVλ^*; λ^N, pSV-CMVλ^N.
[b]Immunofluorescence assays were performed on stable transfectant clones except for COS cell transfectants which were assayed in transient.
[c]J558L constitutively express the secretory light λ chain (Oi *et al.*, 1983).
[d]C and N indicate cytoplasmic and nuclear fluorescence respectively. Part A refers to the cytoplasmic constructs, whereas part B refers to the constructs for nuclear targeting.

of both Ac38 ad Ac146 antibodies to the anti-NP antibody is specifically blocked by a polyvalent hapten−protein conjugate (Reth *et al.*, 1979).

Indirect immunofluorescence with the anti-idiotypic antibodies Ac38 and Ac146 was carried out on fixed and permeabilized cells (Figure 4). Clear staining was obtained with C6λ*μ* (Figure 4B) demonstrating that the mutant heavy and light chains associate in the cytoplasm. As expected for a protein with a cytoplasmic localization, the fluorescence obtained with C6λ*μ* cells was diffuse and did not show the clear-cut exclusion of the cell nucleus, nor the punctate staining typical of the distribution of the secretory antibodies in C6λμ (Figure 4A; Cattaneo, 1988). The uniform dispersion of the assembled antibody molecules throughout the cytoplasm and the karyoplasm reflects the analogous distribution of the individual chains (Figure 2A and C). The two anti-idiotypic antibodies Ac38 ad Ac146, which recognize independent epitopes on the associated variable regions of the NP antibody, gave identical results. In all cases tested double transfectants showed a diffuse staining when assayed with either anti-idiotypic or anti-λ antibodies (Table I). The staining with anti-idiotypic antibodies was dependent on both μ and λ polypeptide chains being present within the same compartment of the cell (Table I). Thus, anti-idiotypic antibodies do not stain J558L cells (which constitutively express the secretory λ light chain of the NP antibody) transfected with pSV-HSμ*, as the cytosolic μ* heavy chains are in a different compartment from the secretory light chains of the plasmacytoma host; however staining is obtained with the J558L [pSV-CMVλ*/pSV-HSμ*] double transfectant. Similarly, immunoprecipitation studies with J558L[pSV-HSμ*] cells show that secretory λ light chains do not coprecipitate with the cytoplasmic heavy chain (R.Sitia, personal communication). These results confirm that association of free heavy and light chains during extraction procedures is unlikely. All

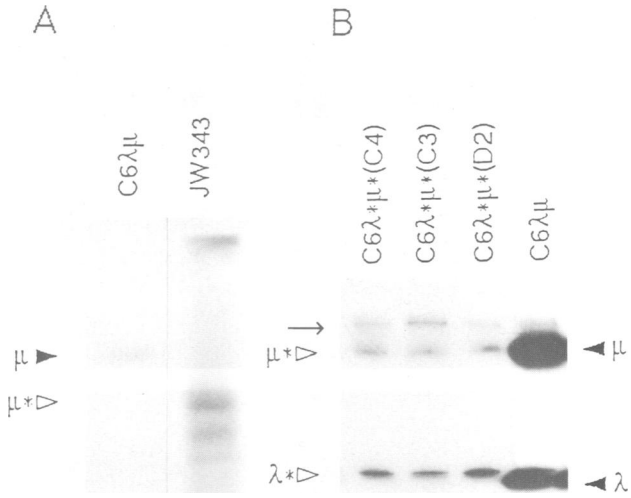

Fig. 3. Analysis for the presence of intracellular heavy and light chains. A rabbit antiserum against mouse μ heavy chain (**A**) and against mouse λ light chain (**B**) was used to precipitate the heavy and light chain from cell extracts after heat induction at 43°C for 2 h, incubation at 37°C for 15 h, and biosynthetic labelling with [^{35}S]-methionine for 2 h. Immunoprecipitated proteins were analysed by SDS−PAGE using 7% acrylamide in A and 15% in B. (**A**) **Lane 1**, C6λμ cells; lane 2, J558L plasmacytoma cells transfected with pSV-HSμ*. (**B**) *Lanes 1−3*, different clones of C6 glioma cells transfected with pSV-HSμ* and pSV-CMVλ*; **lane 4**, C6λμ. The difference in mol. wt between the secretory and the cytoplasmic μ chains cannot be seen in this experiment due to the high percentage of acrylamide used. Arrowheads point to the secretory (μ) and cytoplasmic (μ*) heavy chain and to the secretory (λ) and cytoplasmic (λ*) light chain. The uppermost band (arrow) in B is the origin of the gel.

the results summarized in Table I were observed with stable clones (except for COS cells which were assayed transiently). However, as previously found in C6λμ cells (data not shown), only ~15−20% of the cells in the population were stained in the immunofluorescence assays (discussed below).

Targeting of cytosolic antibodies to the cell nucleus

Plasmids pSV-CMVλ^N and pSV-HSμ* were transfected into several cell lines and stable clones were analysed by immunofluorescence for the expression and intracellular localization of the mutant immunoglobulins (Table IB). Staining of stable transfectants of CS simian fibroblast cells with anti-idiotypic antibodies (Figure 5B) lit up cell nuclei very clearly, revealing also the cytoplasm more weakly. All the positive cells in the population (~5−10%) showed such nuclear staining. As the anti-idiotypic antibodies only recognize the combination of V_H and V_L, the results suggest that the μ* and λ* chains have assembled in the cytoplasm prior to translocation into the nucleus, since only the light chain contained a nuclear localization signal. Alternatively, the light chain accumulated in the nucleus might form a nuclear 'trap' for the uniformly distributed heavy chain molecules.

The antibody molecules targeted to the nucleus retain their antigen-binding properties, as also shown by competition experiments in which cells fixed and permeabilized were preincubated with antigen (NP coupled to bovine serum

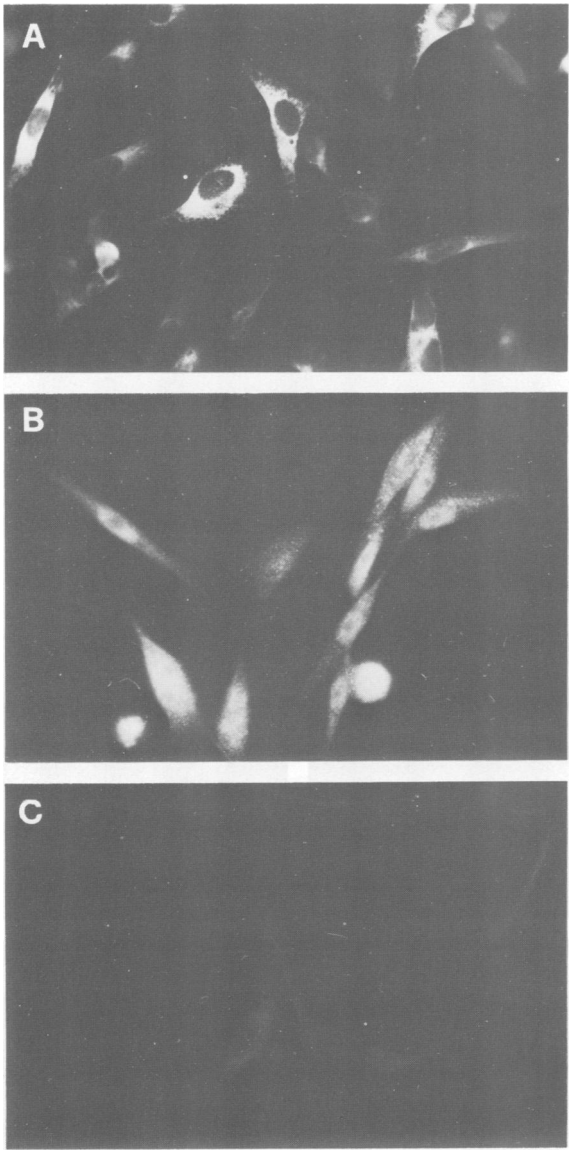

Fig. 4. Immunofluorescence of C6[pSV-HSμ*/pSV-CMVλ*]glioma transfectants. (**A**) C6$\lambda\mu$ cells. (**B**) C6 glioma cells transfected with pSV-HSμ* and pSV-CMVλ* (C6λ*μ*, clone D2). (**C**) Untransfected C6 glioma cells. Staining was with the monoclonal antibody Ac38; identical results were obtained with the monoclonal antibody Ac146.

albumin) prior to challenge with anti-idiotypic antibodies (Figure 5C).

Immunofluorescence was also carried out on parallel cultures using antibodies recognizing the λ and μ constant domains. Figure 5E and F shows cells in which nuclei are lit up by anti-λ antibodies, with a staining similar to that revealed by anti-idiotypic antibodies. Interestingly, however, at variance with the results obtained wtih COS cells (Figure 2B), only a proportion of the positive cells displayed the strongest signal in the nucleus (Figure 5E and F), the other cells showing an intense perinuclear distribution, along with a weaker dot-like staining visible within the nucleus itself (Figure 5G).

Staining with anti-μ antibodies gave a weak cytosolic diffuse distribution, without showing a particular enrichment in the nuclei (not shown). The weakness of this staining is in keeping with the short half-life of the cytoplasmic heavy chain polypeptide (Sitia *et al.*, 1987). In contrast, the fluorescence obtained with anti-λ antibodies is much more intense, suggesting a longer half-life of the polypeptide carrying the nuclear localization signal.

The expression of the transfected immunoglobulin genes was analysed in several different host strains (Table IB). In all host lines transfected with pSV-CMVλ^N it was confirmed that anti-λ antibodies gave an immunofluorescence signal much more intense than in the cells expressing the cytoplasmic λ* light chain. It should be noted, moreover, that only simian cells, but not rodent cells, transfected with pSV-CMVλ^N and pSV-HSμ* showed nuclear staining with anti-idiotypic antibodies. This is considered below.

These results confirm that in all cell lines tested, heavy and light chains can assemble in the cell cytoplasm, and also show that nuclear targeting of functional antibodies can occur in some cell types.

Discussion

This paper describes experiments designed to explore the potential of a new means of selectively perturbing specific intracellular functions, based on the stable cytoplasmic expression in mammalian cells of suitably modified immunoglobulin DNA sequences. Plasmids were constructed to allow the expression of a hapten-specific immunoglobulin in the cytoplasm of non-lymphoid transfectants. An important question which needs to be answered is whether the heavy and light chains of an antibody can associate functionally, once targeted to the cytoplasm by a modification of their signal sequences. A corollary of this is to ask whether it may be possible to target assembled cytosolic antibodies to different intracellular compartments other than the secretory one.

In order to assay the association of heavy and light chains *in situ* we utilized two well characterized anti-idiotypic antibodies which would only recognize idiotypes formed by the association of the heavy and light chain variable regions of the parental antibody (Reth *et al.*, 1979). The results obtained demonstrate the formation of a stable V_H-V_L complex in the cytoplasm of cell transfectants. Thus, the selective block of the function of an intracellular antigen by cytoplasmic antibodies is not inconceivable.

The range of antigens susceptible to *in vivo* neutralization can be extended by including into the DNA coding for the immunoglobulin other intracellular sorting sequences, such as, for instance, nuclear localization signals. Indeed, crosslinking of a synthetic peptide containing the nuclear localization sequence of the large T antigen (PKKKRKV) to immunoglobulin IgG protein followed by microinjection into *Xenopus* oocytes or into the cytoplasm of mammalian cells was shown to result in nuclear accumulation of antibody molecules (Goldfarb *et al.*, 1986; Lanford *et al.*, 1986). We studied the expression of a light chain containing this nuclear localization sequence (substituting the hydrophobic amino acids of the secretory signal sequence) with the expression of a cytoplasmic heavy chain. The results obtained show that it is possible to target assembled antibodies to the cell nucleus.

The experiments described in this paper were mainly designed to demonstrate the feasibility in principle of expressing intracellular antibodies. Although the results obtained are promising in this respect, there are however

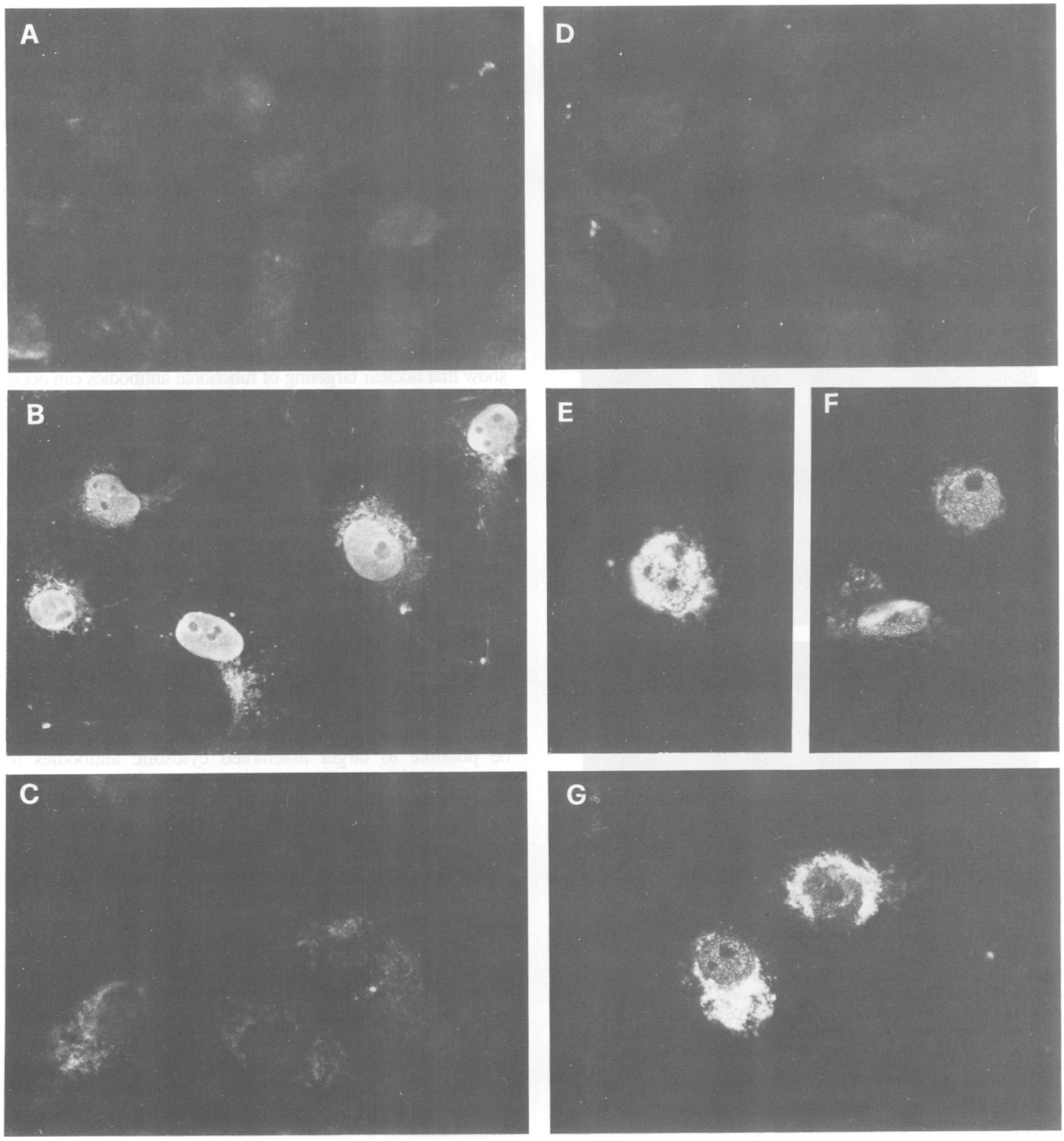

Fig. 5. Immunofluorescence of cells expressing nuclear antibodies. CS (**A, D**) and CS $\lambda^N\mu$* (**B, C, E, F, G**) cells were stained with anti-idiotypic Ac38 antibodies (A, B, C) and with anti-λ antibodies (D, E, F, G). All cells in this experiment were heat shocked 15 h before fixation (see Materials and methods). (**A, D**) CS simian fibroblasts stained with Mab Ac38 (A) and with anti-λ antibodies (D) respectively. (**B**) CS cells transfected with pSV-HSμ* and pSV-CMVλ^N stained with Mab Ac38. Nuclei light up very clearly with the exception of nucleoli. Some staining of the cytoplasm is also visible. (**C**) Same cells and same antibody as in B, except that before addition of the anti-idiotypic antibodies cells were preincubated with 5 μg/ml NP coupled to bovine serum albumin; the nuclear staining by anti-idiotypic antibodies (both Ac38 or Ac146) is abolished. (**E, F, G**) CS$\lambda^N\mu$* cells stained with anti-λ antibodies. Some cells (E, F) show a nuclear staining which is similar to that obtained with anti-idiotypic antibodies, while the remaining positive cells in the population show a cytoplasmic staining which is particularly intense in perinuclear regions and absent in the cell periphery (G). Fluorescence dots are also visible in cell nuclei. This fluorescence pattern is very different from that observed in CS cell transfectants expressing a secretory light chain, in which the cell cytoplasm is uniformly stained and no fluorescence is seen in the nuclei.

some points to be discussed and some aspects which need to be improved.

In view of the reducing environment of the cell cytoplasm, the question arises as to whether the cytosolic heavy and light chains associate via disulphide bridges. Thus, even proteins in which biochemical and structural evidence reveals the presence of juxtaposed cysteine residues (Quinlan *et al.*, 1986) do not show disulphide bridges in the cytoplasm of

living cells. It should be noted, however, that when separated immunoglobulin chains are mixed and brought to neutral pH they reassociate to form an intact Ig molecule even if the original disulphide linkages cannot reform (Kabat, 1968). Therefore, the formation of disulphide bridges between immunoglobulin chains in the cytosol should not represent an absolute requirement for the intracellularly expressed antibodies to be functional, but represents, nevertheless, an interesting point that is presently under investigation.

The half-life of the cytoplasmic antibody chains appears to be very short. This was probably because the amino acid sequence we introduced at the N terminus happens to have (but does not have to have) a composition quite similar to the PEST sequences found at the N terminus of short-lived cytoplasmic proteins (Rogers *et al.*, 1986). The different N terminus sequence of the nuclear light chain appears to lead to a longer half-life (data not shown; also see Table I), suggesting that rapid turnover of cytosolic antibody chains might indeed not be a general problem. Deleting the leader altogether or substituting it with the initial amino acids of a long-lived cytoplasmic protein might help increase the half-life of the cytoplasmic immunoglobulin polypeptides (work in progress).

It has been reported that the expression of the *hps70* gene is notably cell-cycle dependent (Milarski *et al.*, 1986). This dependence is a possible reason for the observed expression of cytosolic antibodies in 15–20% of the culture of cloned stable transfectants, when assayed by immunofluorescence. Consistent with this hypothesis, in synchronized C6λμ cells (expressing heavy and light chain under the control of heat-shock promoter) the percentage of positive staining cells is cell-cycle dependent, in a range between 5% and >50% (compared to 20% in the unsynchronized population) (data not shown). The somewhat lower percentage (5–10%) of positive cells for the nuclear antibodies could be due to additional factors such as the cell-line dependence of the SV40 large T antigen nuclear localization signal in a heterologous context (Fisher-Fantuzzi and Vesco, 1988).

The differences observed in the staining of CS transfectants (Figure 5) by anti-isotype and anti-idiotype antibodies (both in terms of intensity and of distribution) could be interpreted by postulating that (i) some sort of masking of the constant domains is occurring in the nucleus (possibly by some heat-shock protein such as *hps70*), (ii) the observed higher amount of light over heavy chain polypeptide leaves a pool of unassembled light chains or alternatively, (iii) the variable domains might be proteolytically separated from their corresponding constant domains, to yield F_v fragments (Sharon and Givol, 1976) carrying the idiotype and the antigen specificity of the parental antibody as well as the nuclear localization signal. Experiments are in progress to address this issue.

We observed a variable efficiency in nuclear targeting of assembled antibodies among different cell lines; in fact, the immunofluorescence studies carried out with anti-idiotypic antibodies demonstrate that only simian CS and COS cell transfectants show nuclear accumulation while the other cell lines tested (mouse myeloma, mouse fibroblasts and rat glioma) were less efficient in targeting of the antibody to the nucleus. The explanation for this result is likely to be due to the fact that the functioning of the SV40 large T antigen nuclear localization signal, when it is out of its natural amino acid context, is cell-line dependent. Efficient nuclear targeting with such a signal in rodent cells can only be achieved by reiteration of the signal itself (Fisher-Fantuzzi and Vesco, 1988).

A similar study dealing with the expression of intracellular antibodies in yeast cells has been recently described (Carlson, 1988). The DNA sequences coding for a monoclonal antibody directed against the yeast enzyme alcohol dehydrogenase I (ADHI) were mutated in order to allow expression in the cell cytoplasm. At variance with our study, the assembly of the heavy and light chains was not demonstrated *in situ* but evidence was based on (i) a limited neutralization of the enzyme activity in the transfectant cells and (ii) immunoprecipitation of intracellular antibodies from cell extracts. Interestingly, *bona fide* association of heavy and light cytosolic chains (Carlson, 1988) appears to be much more efficient than that observed in the seemingly more favourable case of expression of immunoglobulin sequences in yeast cells as secreted proteins (Wood *et al.*, 1985), possibly due to the presence of the relevant antigen in the cell in the former case. Our finding that addition of the antigen in the extraction buffer increased the proportion of assembled chains suggests that the presence inside the cell of the antigen recognized by the expressed antibody may help in its stabilization as also shown from *in vitro* experiments on recombination of antibody chains (Kabat, 1968).

Monoclonal antibodies against proteins of the secretory apparatus have been previously expressed in *Xenopus* oocytes or in mammalian cells via microinjection of mRNA from the corresponding hybridoma cell line (Valle *et al.*, 1982, 1988; Burke and Warren, 1984; Rosa *et al.*, 1989). Microinjection of hybridoma mRNA or of antibodies (for reviews see Graessmann *et al.*, 1980; Morgan and Roth, 1988) is a method that has several useful features, including the possibility of utilizing antibodies selectively directed to post-translational modifications of intracellular proteins or even to non-protein antigens, something that is not possible with the other methods now available to disrupt the activity of specific proteins in mammalian cells. However there are severe limitations to the applicability of the microinjection technique, which is mainly limited to studies of short-term, fast biological responses which can be assayed in a very small number of cells. On the contrary, the stable expression of immunoglobulin coding sequences described in this paper would allow the disruption experiments to be carried out simultaneously in large populations of cells, without requiring direct physical access to each of them, and on a longer time scale. More suitable forms of the antibodies for intracellular expression might be represented by F_V fragments (Riechmann *et al.*, 1988; Skerra and Pluckthun, 1988), single chain (Bird *et al.*, 1988; Huston *et al.*, 1988) or single domain (Ward *et al.*, 1989) antibodies.

The expression and targeting of engineered intracellular antibodies or antibody domains may be of particular usefulness in cells, such as mammalian and others, where a systematic genetic analysis is not feasible.

Materials and methods

Plasmid construction

Plasmids pSV-HSμ1 and pSV-HSμ* (cytoplasmic leader) (Mason *et al.*, 1988) and pSV-CMVμ (Neuberger and Williams, 1988) have been previously described; plasmid pSV-CMVλ1 was derived from pSV-HSλ1 (Cattaneo and Neuberger, 1987) by substituting the promoter of the *Drosophila* heat-

shock *hsp70* gene with the human cytomegalovirus promoter (Boshart *et al.*, 1985); plasmids pSV-CMVλ* (cytoplasmic leader) and pSV-CMVλ^N (nuclear leader) are derivatives of pSV-CMVλ1 and were obtained by site-directed mutagenesis (Carter *et al.*, 1985) using an M13 subclone containing the region to be mutagenized and the two oligonucleotides 5'CTGTAGC-TGTTTCTTCCGAGGAGCGCCTCTCTTACAGCTCCATC3' (coding for a 'cytoplasmic' signal sequence) and 5'CTGTAGCTGTTCCTCCACCT-ACCTTTCTCTTCTTTTTTGGCTTACAGCTCCATC3' (coding for a 'nuclear' signal sequence) respectively, which were synthesized by T.Smith on an Applied Biosystems machine. Mutagenized M13 clones were transfected into *Escherichia coli* BMH 71−18 mutL (Kramer *et al.*, 1984) and mutations were confirmed by sequencing (Sanger *et al.*, 1977).

Cell lines and transfections

Mouse plasmacytoma J558L (Oi *et al.*, 1983), mouse myeloma P3-X63-Ag8 (Kohler and Milstein, 1975), rat C6 glioma (Benda *et al.*, 1968), rat C6λμ glioma (Cattaneo and Neuberger, 1987), mouse Ltk$^-$, simian COS and CS (Fisher-Fantuzzi and Vesco, 1988) cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum. CS cells were a kind gift from L.Fisher-Fantuzzi. COS cells were transiently transfected in the presence of DEAE−dextran as described (Pelham, 1984). All other cell lines, except for CS cells, were transfected by electroporation (Neumann *et al.*, 1982). Briefly, 10^7 cells were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended for 5 min in 250 μl of ice-cold PBS containing 10 μg of plasmid DNA. Cells were then subjected to 15 pulses (1 s apart) of the appropriate voltage (varying between 1.5 kv and 2.2 kV/0.5 cm depending on the cell line). CS cells were transfected by the polybrene method (Chaney *et al.*, 1986). About 4×10^5 cells in 6 cm diameter dishes were incubated with 1−5 μg of the relevant plasmid DNA. For transient expression, cells were harvested and analysed 48 h after transfection. To isolate stable transfectants, cells transfected with the neomycin resistance marker were selected by growing them in 1 mg/ml of Geneticin (G418) (Sigma) 48 h after the transfection. After 1 week selection with G418, cultures which had also received plasmids carrying a guanosine-phosphoribosyl-transferase (*gpt*) selectable marker, were further selected with mycophenolic acid at a concentration of 5 μg/ml as described by Mulligan and Berg (1982).

Analysis of proteins

Heat shocking of cells (when required), biosynthetic labelling, cell extraction and immunoprecipitations were carried out as described (Cattaneo and Neuberger, 1987). Rabbit anti-λ light chain antibodies and rabbit anti-μ heavy chain antibodies were from Miles, and Protein A−Sepharose was from Pharmacia. SDS−PAGE was performed according to Laemmli (1970).

Immunofluorescence

Cells were plated on poly-L-lysine coated multi wells slides (Flow Laboratories), fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1 M Tris−Cl pH 7.4 and 0.2% Triton X-100. When required, cells were heat-shocked for 45 min at 42.5°C 16 h before their fixation (these heat induction conditions are milder than those used for immunoprecipitation experiments, to preserve adhesion of cells to the substratum). Cells were then washed in PBS and incubated for 2 h at room temperature with the appropriate dilution of the first antibody. Biotinylated anti-mouse λ, biotinylated anti-mouse μ and streptavidin−fluorescein were purchased from Amersham International. Fluorescein-conjugated goat anti-mouse Ig was from Nordic. Hybridoma cell lines producing monoclonal antibodies Ac38 and Ac146 (Reth *et al.*, 1979) were a kind gift of Prof. K.Rajewski.

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