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Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin

Joe Sambrook, Linda Rodgers, Judy White [1,2] and Mary-Jane Gething

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724, and [1]Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

[2]Present address: Department of Pharmacology, University of California, San Francisco, CA 94143, USA

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We have developed and characterized several murine cell lines that constitutively express either the full-length, membrane-bound form of influenza virus hemagglutinin (HA) or a truncated version of the protein (HA^{sec}) that lacks the carboxy-terminal anchoring sequences and is secreted from cells. cDNAs encoding HA or HA^{sec} were linked to the murine metallothionein-I promoter or the SV40 early promoter, and inserted into plasmids containing the transforming DNA fragment of bovine papilloma virus (BPV). The resulting vectors were introduced into three cultured lines of murine cells – C127, NIH3T3 and MME – either alone or in the presence of a plasmid that carries the aminoglycoside transferase gene of Tn5. The resulting lines of MME cells contained 1 – 5 copies of the vector in an integrated state and expressed low levels of HA (~ 10^4 molecules/cell). In contrast, lines of C127 and NIH3T3 cells were obtained that express up to 5 x 10^6 molecules of HA per cell or secrete ~ 10^7 molecules of HA^{sec} per cell per 24 h. Some of these cell lines carry multiple (30 – 200) copies of the vector in an integrated state; in others, the vector is propagated as unit-length episomes or as oligomers. Both the membrane-bound and secreted forms of HA expressed in these cell lines display a more extensive pattern of glycosylation than HA or HA^{sec} synthesized in simian cells and they are transported to the cell surface more slowly. Pulse-chase experiments suggest that the step which limits the rate at which HA and HA^{sec} travel down the secretory pathway occurs in the rough endoplasmic reticulum before the molecules are transferred to the Golgi apparatus. Using indirect immunofluorescence in combination with a cell sorter, we have shown that the level of expression of HA within cloned populations of producing cells can be variable. However, >90% of the cells in certain cell lines display considerable quantities of HA on their surface, as judged by their ability to bind red blood cells in large numbers. We have taken advantage of the membrane fusion activity of HA to effect the fusion of erythrocytes to these cells and to deliver the contents of red cell ghosts into the cells' cytoplasm.

Key words: hemagglutinin/BPV/mammalian vectors/cell fusion/secretion

Introduction

In recent years, the hemagglutinin (HA) of influenza virus has been used increasingly as a model to study the biosynthesis, modification and transport of integral membrane proteins in mammalian cells. The groundwork for this approach was laid when it was shown that the cloned gene for HA could be expressed from eukaryotic vectors with very high efficiency in cultured mammalian cells; furthermore the newly-synthesized HA was translocated through the endoplasmic reticulum and transported *via* the Golgi apparatus to the cell surface along a pathway indistinguishable from that taken by a large number of authentic cellular membrane proteins (reviewed in Gething and Sambrook, 1983).

These initial findings were extended to include a number of mutant and chimeric forms of HA, in which the DNA sequences coding for the putative hydrophobic signal and anchor regions of the protein were deleted, mutated at specific sites or attached to sequences coding for other proteins (Gething and Sambrook, 1982a, 1983; Doyle *et al.*, 1984; Gething *et al.*, 1985). Analysis of the expression of the resulting constructs has led to the following conclusions. (i) The hydrophobic amino-terminal sequences of HA are both necessary and sufficient to cause translocation of the newly-synthesized protein across the membrane of the rough endoplasmic reticulum. (ii) The hydrophobic carboxy-terminal sequences of HA are sufficient to anchor the completed molecule in the plasma membrane; deletion of these sequences results in secretion of a truncated HA from the cell. (iii) The hydrophilic cytoplasmic tail of HA can tolerate considerable change in amino acid sequence and composition without affecting the biosynthesis or transport of the completed molecule. However, certain alterations to this region of the molecule drastically lower the rate at which HA travels from its site of synthesis to the Golgi apparatus: other alterations affect transport of HA from the Golgi to the cell surface.

Virtually all of this work has been carried out with vectors based on SV40 that express extremely large quantities of HA during the course of a lytic cycle of viral growth in permissive simian cells. However, for a number of reasons, it would be useful also to have available permanent lines of cells that express wild-type or mutant HA molecules but are not destined to die as a consequence of viral infection. Such cells would provide a convenient and continuous source of protein for use in structural studies or as an immunogen and could serve as priming or target cells in studies of cell-mediated cytotoxicity (Braciale *et al.*, 1984). Furthermore, cell lines that constitutively express large quantities of biologically active HA on the plasma membrane would be expected to bind and fuse with erythrocytes and thus serve as universal recipients for materials that have been loaded into red cell ghosts. Finally, such cells would provide the appropriate material from which to derive, by selection or screening, a series of mutant cells that are defective at one stage or another of biosynthesis or transport of membrane proteins in general. Cell lines developed by standard co-transformation techniques (Perucho *et al.*, 1980), contain 2 – 10 copies of the HA gene and express HA in amounts sufficient to be detected by cell-mediated cytotoxicity (Braciale *et al.*, 1984), but not by hemadsorbtion

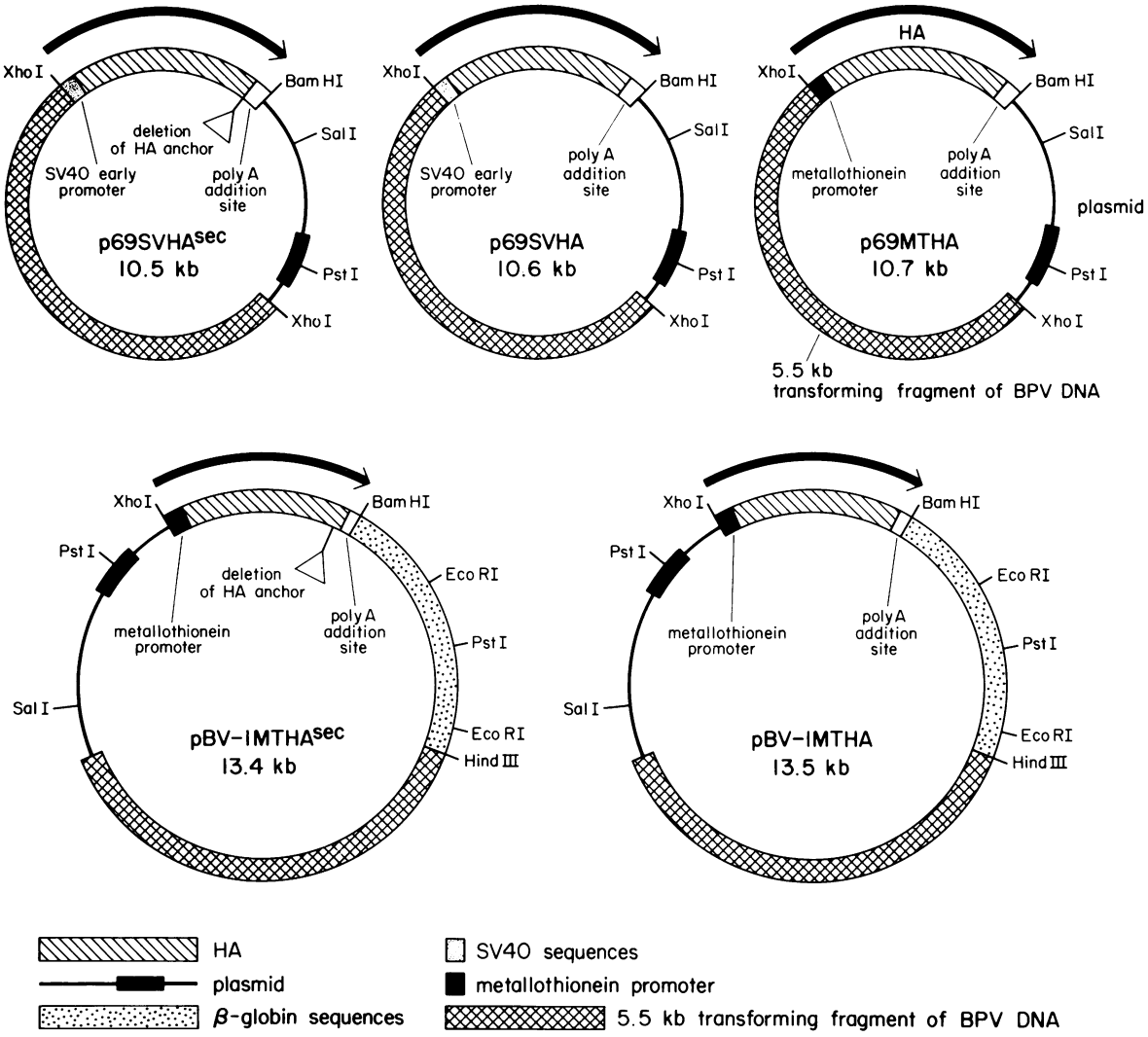


Fig. 1. Vectors used to develop BPV-HA transformed cell lines. The plasmids and the details of their construction are described in Materials and methods.

or cell fusion.

We therefore decided to investigate the use of vectors based on bovine papilloma virus, which have previously been used to generate cell lines that synthesize large quantities of a number of secretory or membrane proteins [for example, β-interferon, (Zinn *et al.*, 1982; Mitriani-Rosenbaum *et al.*, 1983): human growth hormone, (Pavlakis and Hamer, 1983): hepatitis virus surface antigen, (Wang *et al.*, 1983; Stenlund *et al.*, 1983; Hsiung *et al.*, 1984): and HLA heavy chain (DiMaio *et al.*, 1984)]. Bovine papilloma virus (BPV) is a small DNA virus that is capable of morphologically transforming certain types of murine cells. Vectors containing the entire BPV genome or 5.5-kb subgenomic transforming fragment are in many cases propagated as stable multicopy (20 – 100 copies/cell) extrachromosomal elements in transformed cells (see, for example Law *et al.*, 1981; Sarver *et al.*, 1981; DiMaio *et al.*, 1982; Zinn *et al.*, 1982, 1983). In other cases, the vector sequences are maintained as oligomeric plasmids, or as head-to-tail tandem arrays integrated into the cellular DNA (DiMaio *et al.*, 1984; Pavlakis, personal communication). Whatever the state of the vector DNA, the cell lines invariably carry many copies of the gene of interest: it is this high copy number which is responsible at least in part for the efficient expression of the foreign protein.

This paper describes the derivation and properties of murine cell lines that express HA from recombinant bovine papilloma virus-HA genomes that can be maintained in high copy number.

Results and Discussion

Isolation of cell lines expressing hemagglutinin from BPV-HA recombinant vectors

Three established and well-characterized lines of murine cells were chosen for these studies: C127, because they alone amongst such cells can be morphologically transformed with high efficiency by bovine papilloma virus DNA; NIH3T3, because of their general competency in taking up DNA; and MME, because they form polarized, epithelial sheets in which the apical and basolateral membranes of individual cells are physiologically and structurally differentiated. MME cells however, are not sensitive to morphological transformation by BPV and are not efficiently transfected by DNA.

BPV-HA vectors (Figure 1) were constructed that contain either the complete coding region of the HA gene or a truncated version which lacks the sequences coding for the hydrophobic anchor near the carboxy terminus of the protein. The full length gene codes for a membrane-bound protein that is

Table I. Properties of murine cell lines transformed with BPV-HA recombinant vectors

Cell line	Selection	Screen	Fraction of HA producers	State of DNA in cells	Molecules/cell (best producers)	Inducibility by Zn
69-SVHA/C127	T*	RIA	7/33	integrated	2.2×10^4	nr
69-SVHA^{sec}/C127	T*	RIA	17/44	integrated	3×10^4	nr
69-MTHA/C127	T*	rbc/RIA	4/12	integrated	4×10^6	no
BV1-MTHA/C127	T*	RIA	34/48	episomal monomers and oligomers	1×10^6	no
BV1-MTHA^{sec}/C127	T*	RIA	31/48	episomal monomers and oligomers	9×10^6	no
BV1-MTHA/NIH	neo^r	rbc/RIA	22/24	episomal oligomers and integrated	2×10^6	no
BV1-MTHA^{sec}/NIH	neo^r	RIA	9/25	episomal oligomers and integrated	1×10^6	no
BV1-MTHA/MME	neo^r	rbc/RIA	11/24	integrated	1×10^4	8 – 10x
BV1-MTHA^{sec}/MME	neo^r	RIA	49/117	integrated	1×10^4	2x

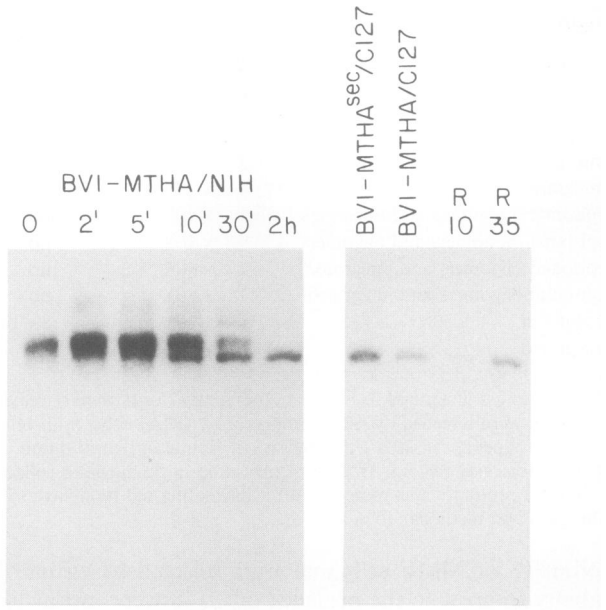
Murine cell lines (C127, NIH3T3 or MME) were transfected with the various BPV-HA vectors illustrated in Figure 1. Individual clones were selected either on the basis of transformed morphology (T*) or by resistance to G418 (neo^r). The clones were screened by radioimmune assay (RIA) either with or without prior selection by red blood cell binding (rbc). The state of the vector DNA in the HA-producing cell lines was analysed by Southern hybridization. HA production was measured by RIA against a standard of purified HA and calculated as molecules per cell. HA^{sec} was measured in the medium collected from cells after a period of 24 h. Inducibility of HA production from the metallothionein promoter was measured after incubating cell monolayers in medium containing 1 – 5 uM $ZnCl_2$. All procedures and assays are described in Materials and methods.

displayed at the cell surface in a fully functional state (Gething and Sambrook, 1981): the truncated version of the gene codes for a secreted form of the protein (Gething and Sambrook, 1982a). Transcription of the HA gene in BPV vectors was controlled by either the SV40 early promoter or the murine metallothionein-I promoter (Hamer and Walling, 1982). To ensure that the resulting transcripts would be efficiently terminated, the HA sequences were inserted so that they lay upstream of a poly(A) addition signal derived from the distal end of the early region of SV40 (nucleotides 2770 – 2533). In addition, all of the vectors carry: (i) the region of bovine papilloma virus DNA (the 69% *Hind*III-*Bam*HI fragment) that codes for functions responsible for morphological transformation (Lowy *et al.*, 1980) and for maintainance of the viral DNA in an extrachromosomal state (Lusky and Botchan, 1984) (ii) plasmid sequences that allow the vectors to be propagated episomally in *Escherichia coli*. In addition, certain vectors (those of the BV-1 series) carry an additional fragment of DNA derived originally from the human β-globin gene cluster. In some systems (DiMaio *et al.*, 1982; Zinn *et al.*, 1983), but not all (DiMaio *et al.*, 1984), this fragment appears to carry an activity that enhances the ability of BPV vectors to replicate as episomes.

C127 cells were transfected with various BPV-HA vectors (p69SVHA, p69SVHA^{sec}, p69MTHA, pBV1-MTHA, pBV1-MTHA^{sec}, shown in Figure 1) and foci of morphologically-transformed cells (which arose at a frequency of ~100 foci/μg of plasmid DNA) were isolated 2 weeks later. Individual foci were recloned and expanded into cell lines. NIH3T3 and MME cells were transfected with a mixture containing the appropriate BPV-HA vector together with plasmid pON_3 which contains a composite gene composed of the SV40 early promoter and the aminoglycoside phosphotransferase gene of Tn5. Expression of the neo^r gene renders mammalian cells resistant to the antibiotic G418. Colonies that grew in the presence of the antibiotic were expanded into cell lines and screened directly by radioimmune assay or by binding of erythrocytes for the production of either secreted or cell-associated HA. The results of the initial screening of some 475 individual cell lines are shown in Table I.

Approximately one-third of the cell lines tested (185/475) expressed detectable quantities of HA ($>5 \times 10^3$ molecules/cell). The proportion of colonies that expressed HA was not affected by the method of selection. Thus, colonies of NIH3T3 or MME cells that were selected by virtue of their ability to grow in the presence of G148 were just as likely to express HA as colonies of C127 cells selected on the basis of their transformed morphology. BPV-HA vectors therefore seem to work as efficiently when they are introduced into cells by unlinked co-transformation as when they are used as the inducers of morphological transformation. The amount of HA produced by different colonies varied up to 10-fold between sibling colonies picked from the same transfection or co-transfection. The reason for this colony-to-colony variation was not investigated. However, the data shown in Table I reveal two factors – the promoter used to drive transcription of the HA gene, and the type of cell transfected – that greatly affected the amount of HA produced. Expression of HA from vectors containing the murine metallothionein promoter was consistently higher (by a factor of 10- to 50-fold) than from the equivalent vectors in which synthesis of HA was controlled by the SV40 early promoter. When production from different cell types were compared, MME cells were the worst producers: despite diligent searching, we were unable to isolate lines of these cells that synthesized constitutively $>10^4$ molecules of HA or HA^{sec} per cell. Both C127 and NIH3T3 cells were capable of producing 10- to 100-fold more HA than MME cells and it was relatively easy to isolate lines of each type of cell that synthesized $>10^6$ molecules of surface-bound HA per cell or $\sim 10^7$ molecules of secreted HA per cell per 24 h.

The transcriptional activity of metallothionein promoters is increased after treatment of cells with heavy metals (Karin *et al.*, 1981; Durnam and Palmiter, 1981; Hamer and Walling, 1982). This regulation is maintained when exogenous metallothionein genes are introduced into cultured cells on a BPV-derived vector and maintained in those cells in an episomal state (Karin *et al.*, 1983). Several hybrid genes, consisting of the promoter and control region of the murine metallothionein-I gene fused to protein-coding sequences have been reintroduced into cultured cells in BPV-derived vectors. In some cases (e.g., human growth hormone, Pavlakis and Hamer, 1983) but not all (e.g., hepatitis virus surface antigen, Hsiung *et al.*, 1984) expression of these hybrid genes is increased by exposing the BPV-transformed cells to heavy metals such as cadmium and zinc. The BV1-MTHA/C127 and NIH3T3 cell lines constitutively produced large quantities of HA in the absence of induction and



SOURCE OF DNA	TREATMENT WITH XHOI AND LIGASE	AMPICILLIN-RESISTANT COLONIES
69-SVHA/C127	-	0
69-SVHA/C127	+	0
BV1-MTHA/C127	-	2.1×10^3
BV1-MTHA/C127	+	2.9×10^4
BV1-MTHA/NIH	-	1.0×10^2
BV1-MTHA/NIH	+	4.6×10^3

Fig. 2. Analysis of the physical state of HA-BPV genomes in transformed cell lines. **Top left:** low mol. wt. DNA was isolated from BV1-MTHA/NIH cells by the procedure of Hirt (1967). The DNA was digested with *Xho*I at 37°C for the times shown. The cleaved DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose by the Southern procedure and then hybridized with nick-translated HA cDNA. **Top right:** Hirt DNA was isolated from the indicated cell lines, digested to completion with *Xho*I and analysed as described above, together with amounts of *Xho*I linearized pBV1-MTHA plasmid DNA which represent 10 and 35 copies of the HA gene per diploid mouse genome. **Lower panel:** DNA isolated from the indicated cell lines was extracted by the Hirt procedure, further purified by extraction with phenol and concentrated by precipitation with ethanol. RNA was removed by digestion with pancreatic RNase (1 μg/ml) for 60 min at 37°C. The DNA was extracted with phenol:chloroform (50:50) and again concentrated by precipitation. An aliquot of the DNA was used to transform *E. coli* strain DH-1 to ampicillin resistance as described by Hanahan (1983). The remainder of the DNA was digested with *Xho*I (which cleaves the input plasmid DNA once), purified as above and incubated at 4°C with T4 DNA ligase. The ligation mixture was used to transform *E. coli* as described above. The numbers represent the number of ampicillin-resistant colonies obtained with DNA extracted from 2 x 10^7 cells.

showed no increase in expression of HA when the cells were incubated for 8–24 h in media containing cadmium or zinc (1–5 μM). In contrast, HA production in one line of BV1-MTHA/MME cells increased at least 10-fold when the cells were incubated in media containing heavy metals (see Table I). The constitutive level of HA production in this cell line is very low: even after induction, the rate of HA synthesis is still considerably less than that in many uninduced lines of BV1-MTHA/C127 and BV1-MTHA/NIH cells. The reasons for these differences in behavior may be related to the number of copies of the vectors in the various cell lines. All the cell lines that constitutively synthesize large quantities of HA contain at least 30 copies of the vector DNA: induction by heavy metals is poor. In contrast, the single inducible cell line contains only a few (~5) integrated copies of the vector DNA. These results are consistent with the hypothesis that the activity of the metallothionein-I promoter in uninduced murine cells is controlled by a diffusible repressor whose effects are neutralized when many copies of the promoter are present in the cell.

From the cell lines transfected with each class of vector, the clone that expressed HA with the greatest efficiency was chosen for further study.

The state of BPV-HA DNA in lines of cells that produce HA

We used Southern hybridization to analyze DNA extracted from lines of cells producing HA. Two different extraction methods were used – one which yields predominantly high mol. wt. DNA (Wigler *et al.*, 1979) and another, the Hirt (1967) procedure, which yields DNA of predominantly low mol. wt. Sequences homologous to the original plasmids were detected in DNAs extracted by both procedures from lines of NIH3T3 and C127 cells transfected with plasmids of the BV1 series. Normally, this result might be taken as evidence for the existence of both integrated and episomal forms of the input DNA. However, the amount of hybridizing material in the Hirt supernatants made from a single cell line varied from preparation to preparation. Furthermore, DNAs prepared by both procedures yielded identical sets of hybridizing fragments when digested with restriction enzymes (data not shown). These results indicate that the BPV-HA plasmids exist in these cells largely as episomes which are extracted relatively inefficiently into Hirt supernatants. Whether these cell lines also carry some BPV-HA sequences in an integrated state is not known.

The only cell lines containing BPV-HA DNA that co-migrated with superhelical or nicked circular input plasmid were those derived from transfection of C127 cells with recombinant plasmids of the BV1 series (Figure 2). Typically, these cells contained between 50 and 120 unit-length episomal copies of the input plasmid per diploid mouse equivalent. In some lines of the BV1/C127 series and in all of the lines of the BV1/NIH series, a smear of strongly-hybridizing material was detected that migrated considerably more slowly than the superhelical form of the original plasmid DNA and slightly slower than the nicked circular or linear forms of the plasmid. Partial digestion of this DNA with *Xho*I (an enzyme that cleaves the input plasmid once) generated full-length linear molecules as well as linear dimers and trimers. Complete digestion generated unit-length, linear molecules (Figure 2). Reconstruction experiments show that the BV1/NIH cells contain between 30 and 200 copies of the input plasmid per diploid mouse equivalent. These results indicate that BV1-HA recombinant plasmids are carried in C127 cells chiefly as unit-length episomes although tandemly-arranged oligomers or catenates are also detectable; in NIH3T3 cells the oligomers or catenates are the predominant form. Such complicated forms have been observed previously in certain lines of C127 cells morphologically transformed by BPV (Law *et al.*, 1981).

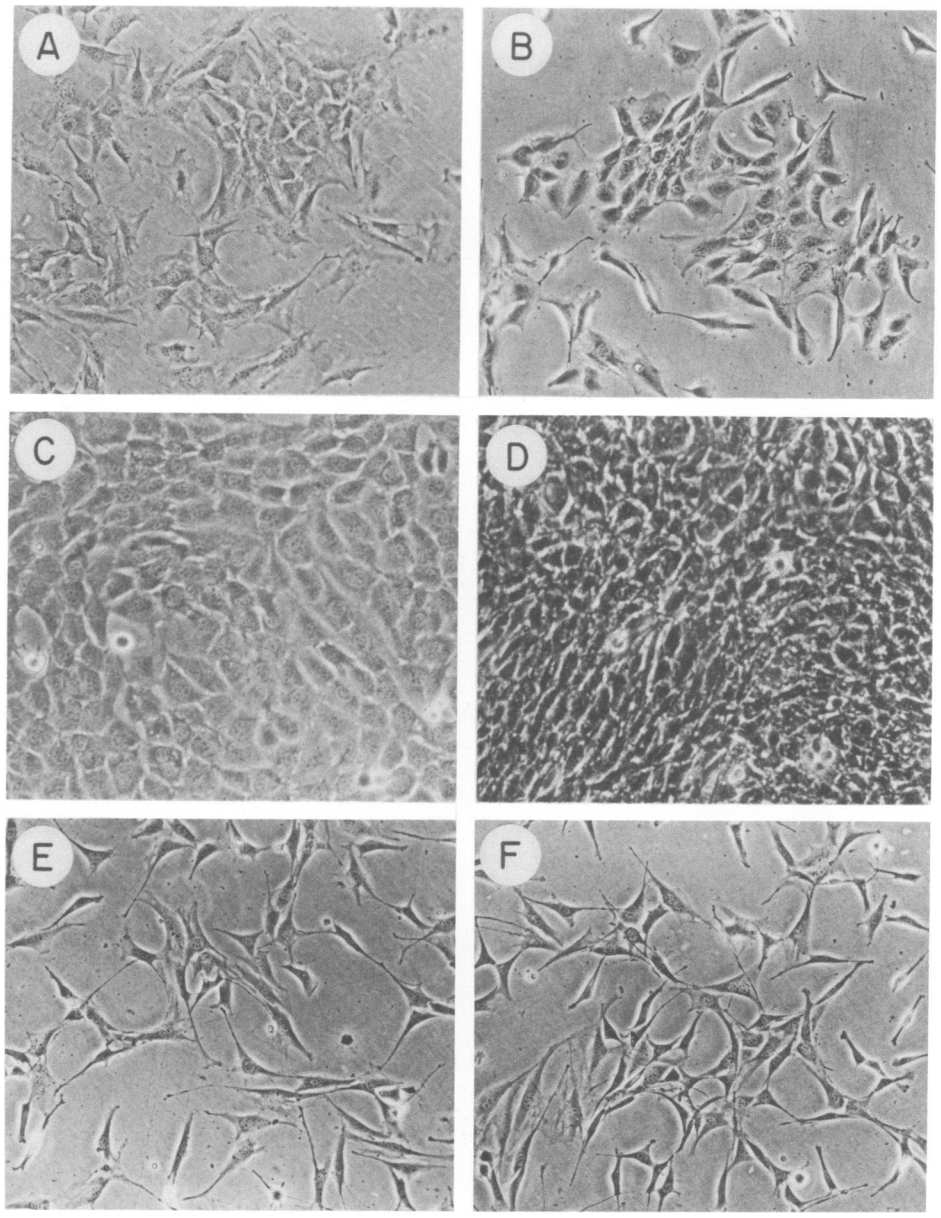


Fig. 3. Effect of transformation with BPV-HA vectors on cell morphology. **Panels A** and **C**, C127 cells; **panels B** and **D**, 69-MTHA/C127 cells; **panel E**, NIH3T3 cells; **panel F**, BV1-MTHA/NIH cells.

Sequences that hybridized to the input plasmid migrated exclusively with high mol. wt. DNA extracted from BV1-HA/MME cells and from lines of C127 cells transfected with plasmids of the p69 series (data not shown). No hybridizing material was detected in DNA extracted from these cells by the Hirt procedure. We conclude that these cell lines carry BPV-HA recombinant DNAs in an integrated form. From reconstruction experiments we estimate that cell lines of the BV1/MME series contain 5 – 10 copies, and those of the p69/C127 series contain 50 – 80 copies of the input plasmid sequences per diploid mouse genome equivalent.

To confirm that some of the cell lines contained free BPV-HA genomes that were not integrated into the chromosome, we attempted to recover the recombinant plasmids from cell extracts. Low mol. wt. DNA prepared by the Hirt (1967) procedure from cell lines of three different lineages (p69/C127; BV1/C127; BV1/NIH) was used in experiments to transform *E. coli* to ampicillin resistance. The results are shown in Figure 2. We were unable to recover plasmids from low mol. wt. DNA isolated from C127 cells transformed by BPV-HA recombinants of the p69 series – a result consistent with the observation that all of the BPV-HA sequences in these cells are carried in an integrated state. We obtained transformants using DNA isolated from lines C127 and NIH3T3 cells that carry BPV-HA recombinants of the BV1 series. The number of transformants increased some 10- to 40-fold after the low mol. wt. DNA was digested with *Xho*I (to convert catenates or oligomers to unit length linear molecules) and then recircularized by treatment with T4 DNA ligase. We conclude (i) that the higher order forms of the BPV-HA recombinants in these cells are either not competent to transform *E. coli* or do so with very poor efficiency and (ii) that the monomers released by digestion with *Xho*I are capable of transformation. No rearrangements of the input BPV-HA vectors had taken place during transfection or passaging of the cell lines because plasmid DNAs prepared from a number of the transformed

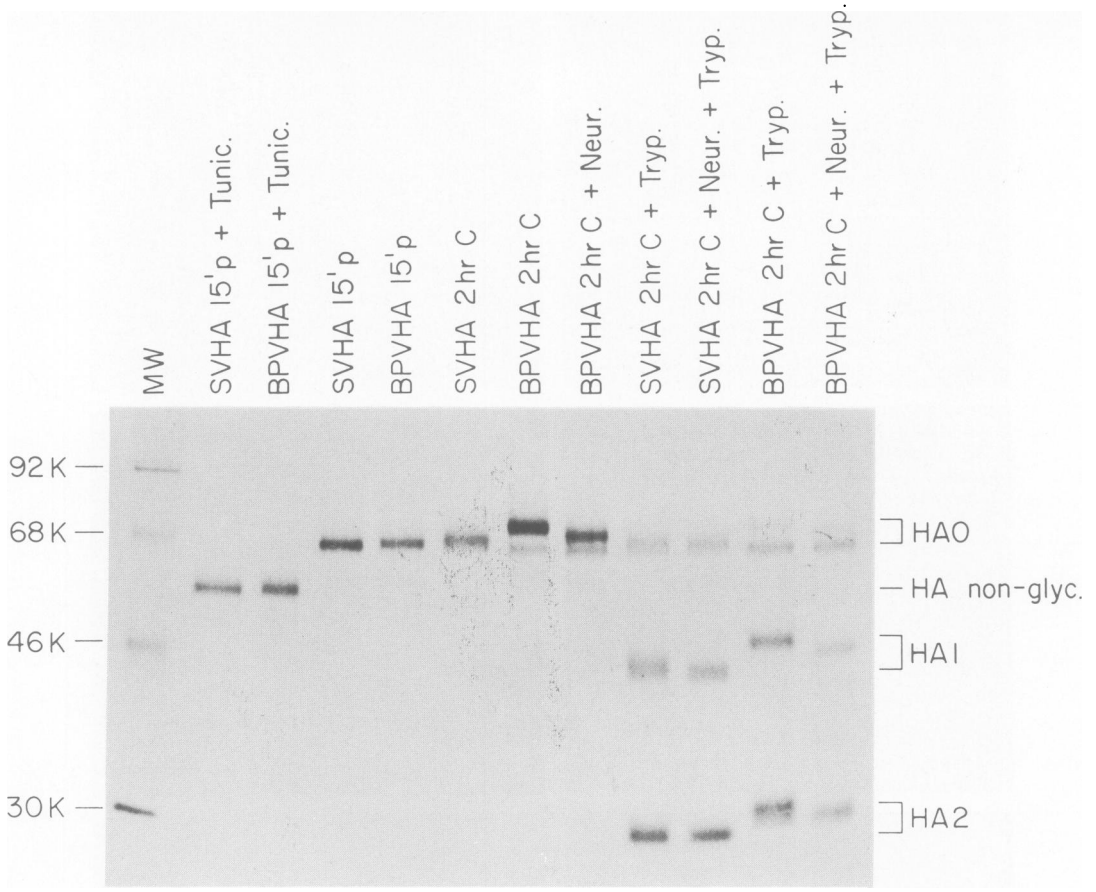


Fig. 4. Comparison of HA synthesized from SV40-HA and BPV-HA vectors. The figure shows an autoradiograph of an SDS-PAGE separation of HA polypeptides immunoprecipitated from [^{35}S]methionine-labelled extracts of cells producing HA from SV40-HA or BPV-HA vectors. The conditions for infection of simian CV-1 cells with SVEHA3 recombinant virus have been described previously (Gething and Sambrook, 1981). The cells were labelled at 42 h post-infection. Cells of the continuous murine line BV1-MTHA/NIH were labelled at ~80% of confluency. Cells were pulsed for 15 min with [^{35}S]methionine (50 μCi per 5 cm dish of infected CV-1 cells or 150 μCi per 5 cm dish of NIH cells) as described previously (Gething and Sambrook, 1981, 1982a) either in the presence or absence of tunicamycin (5 μg/ml). When 2 h chases were performed, the labelling solution was removed, the cells were washed twice before further incubation in complete DME medium. Treatment with trypsin (5 μg/ml in PBS) or neuraminidase (*0.5 units/ml in PBS) was carried out in the final 15 min of the chase period. The preparation of cell extracts, immunoprecipitation with a high-titre rabbit anti-HA serum and SDS-PAGE was performed as previously described (Gething and Sambrook, 1981).

bacterial colonies were identical to the original vectors as judged by digestion with a number of restriction enzymes (results not shown).

Effect of BPV-HA vectors on cell morphology

In contrast to the parental C127 cells, lines of C127 transformed by BPV-HA vectors that express large quantities of HA grow rapidly in medium containing 2% serum. In sparse culture, the parental and transformed C127 cells differ little in morphology (Figure 3, panels A and B). However, in dense cultures the transformed cells grow into heaped criss-crossed layers in which mitotic figures are prominent, while the parental untransformed cells form flat orderly monolayers in which mitotic cells are rarely seen (Figure 3, panels C and D). On the other hand, lines of NIH3T3 cells that express large quantities of HA or HA^{sec} are virtually indistinguishable from the parental cells in their morphology when growing either in sparse culture (see Figure 5, panels E and F) or when confluent (data not shown). The cells, which require high concentrations of serum, grow rapidly (in medium containing 10% serum their doubling time is 18 h) until a confluent monolayer has formed; the rate of cell division then slows dramatically and there is no further increase in cell number. The saturation density of these cell lines is approximately twice that of the parental NIH3T3 cells. We conclude (i) that the production of large quantities of HA does not materially alter the morphology of the cells and (ii) that the presence of large numbers of copies of the transforming region of the BPV genome may slightly affect the growth characteristics of NIH3T3 cells, but is not sufficient to cause them to display a fully transformed phenotype. BV1-MTHA/NIH cells therefore seem to be similar in their properties to the cells that form the 'indistinct foci' observed by Dvoretsky *et al.* (1980) after NIH3T3 cells had been exposed to BPV. Finally, lines of MME cells that produce HA resemble the parental cells in both their morphology and growth characteristics. In addition a number of these lines have maintained the characteristics of a polarized monolayer; they make tight junctions, display electrical resistance and form domes in culture (M. Roth, personal communication).

Biosynthesis of HA in transformed cell lines

To analyze the biosynthesis and properties of the HA expressed in the various cell lines described above, cells were labelled with [^{35}S]methionine and immunoprecipitates of cell extracts and supernatant media were analyzed by SDS-polyacrylamide gel electrophoresis. An example of the data is shown in Figure 4 in which the HA synthesized in BV1-MTHA/NIH cells

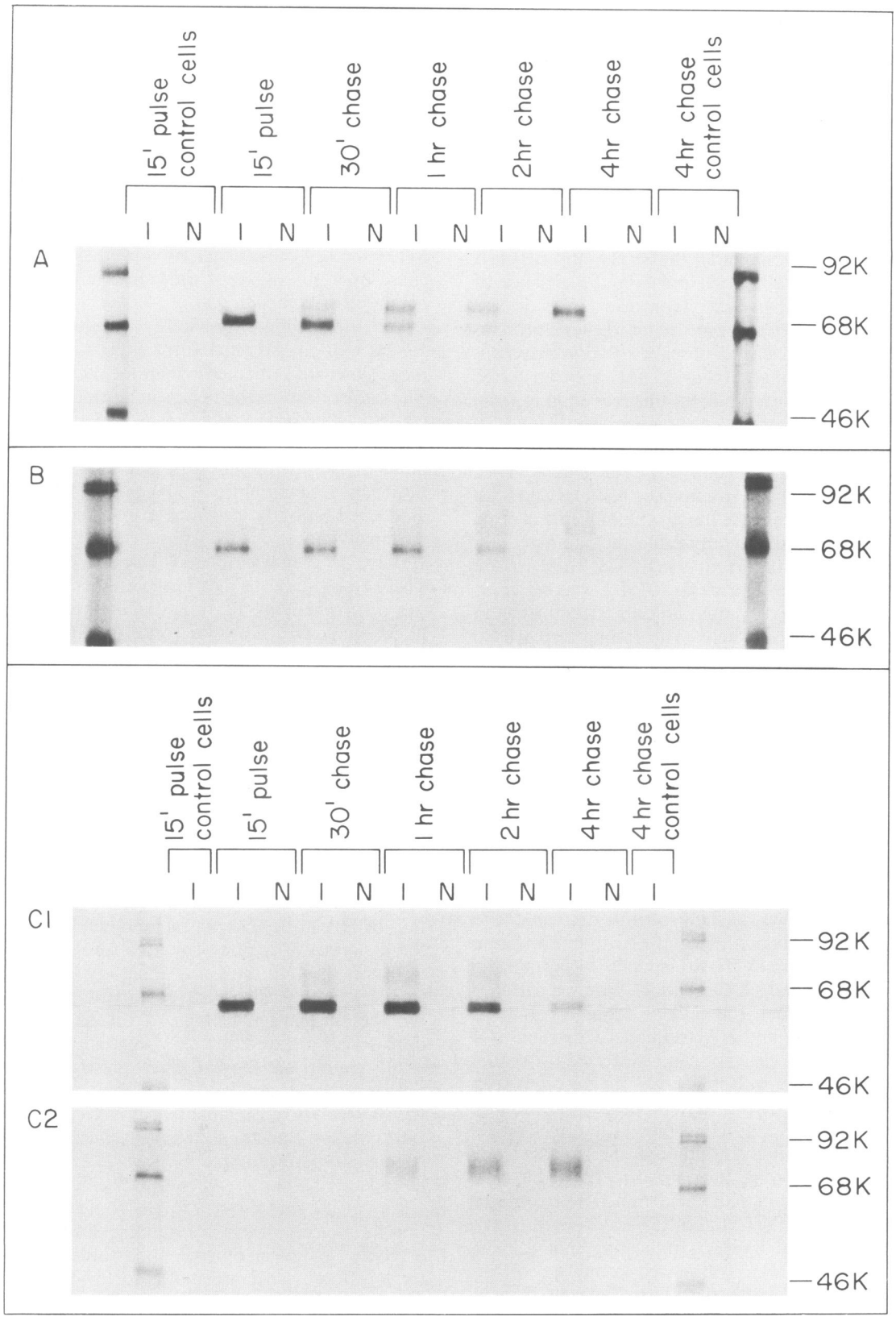


Fig. 5. Time course of intracellular transport of HA in BPV-transformed murine cells. Monolayers of BV1-MTHA/NIH cells **(panel A)**, 69-MTHA/C127 cells **(panel B)**, or 69-MTHA^{sec}/C127 cells **(panel C)** were labelled with [^{35}S]methionine as described in Figure 4 legend. Parent NIH3T3 and C127 cells were included as controls. After varying periods of chase with unlabelled methionine, cell extracts (or supernatent medium, **panel C2)** were collected. The HA polypeptides were immunoprecipitated and separated by SDS-PAGE as described above, I, anti-HA serum; N, non-immune serum.

(BPVHA) is compared with that produced in simian CV-1 cells from an SV40-HA vector (SVHA). In CV-1 cells, the HA expressed from the SV40 vector is identical in size and properties to the protein expressed in an authentic influenza virus infection (Gething and Sambrook, 1981). Figure 4 shows that when cells were labelled in the presence of tunicamycin, a drug which specifically inhibits the addition of the dolichol-oligosaccharide donor and thus prevents primary glycosylation of the polypeptide (Takatsuki *et al.*, 1975), the SVHA and BPVHA proteins were identical in size (mol. wt. 60 000 in this gel system). Similarly, the SVHA and BPVHA proteins labelled during a 15 min pulse in the absence of tunicamycin were indistinguishable from each other in size (mol. wt. 70 000). The difference in size between the proteins synthesized in the presence and absence of tunicamycin is caused by mannose-rich oligosaccharides that are transferred to nascent polypeptides in the endoplasmic reticulum to form a 'core-glycosylated' protein (Neuberger *et al.*, 1972; Waechter and Lennarz, 1976; Hubbard and Robbins, 1979). Further modification of the carbohydrate side chains, which include trimming of glucose and mannose residues and addition of extra sugars, takes place during the movement of the HA glycoprotein from the endoplasmic reticulum to, and through the Golgi apparatus (Hunt *et al.*, 1978; Hubbard and Robbins, 1979; Tabas and Kornfeld, 1979; Tartakoff *et al.*, 1979). This 'terminal glycosylation' results in a further increase in size of the glycoprotein. The results displayed in Figure 4 indicate that the BPVHA protein expressed in NIH3T3 cells differs in its pattern of terminal glycosylation from the HA expressed from the SV40-HA vector (or influenza virus) in CV-1 cells. All of the SVHA labelled in a 15 min pulse has been quantitatively converted during a 2 h chase to the mature, 'terminally-glycosylated' form of 72 000 daltons. By contrast, even after a 2 h chase some residual core-glycosylated BPV-HA remains and the size of the mature form of BPVHA is significantly larger than that of SVHA. Some of this difference in size is caused by sialic acid moieties attached to the oligosaccharide side chains of BPVHA, since the mol. wt. of the protein is decreased after treatment with neuraminidase. This effect can be seen more clearly when the precursor HA0 is split by proteolytic cleavage into its HA1 and HA2 subunits. Murine cell lines, like simian CV1 cells, lack the cellular proteases that can cleave HA0 into its HA1 and HA2 subunits. However, as illustrated in Figure 4, this cleavage can be effected by treating the intact cell monolayers with low levels of exogenous trypsin (5 μg/ml) before cell extracts are prepared for precipitation. Treatment with neuraminidase has little effect on the mobility of the SVHA subunits, although the bands become somewhat sharper, suggesting that a minority of the oligosaccharide side chains contain sialic acid moieties. Even after removal of sialic acid, BPVHA1 and BPVHA2 migrate more slowly than SVHA1 and SVHA2, indicating that the carbohydrate side chains of HA synthesized in murine cells are more complex than those synthesized in simian cells. This increase in complexity may be a result of the slower transport of HA to and through the Golgi apparatus in murine cells (see following section). Similar variation in the composition of the oligosaccharide side chains of HA has been observed when influenza virus is passaged in different cell types (Nakamura and Compans, 1979).

In simian cells lytically infected with SV40-HA recombinant viruses, terminal glycosylation of HA is completed within 20–30 min (Gething and Sambrook, 1982a). However, pulse-chase experiments show that the transition between the core and terminally glycosylated forms of the molecule occurs over a much more protracted period in the murine cell lines that constitutively produce HA (Figure 5). In BV1-MTHA/NIH cells (panel A), only ~10% of the HA labelled in a 15 min pulse with [^{35}S]methionine becomes terminally glycosylated during the next 30 min; a further 3.5 h is required for all of the labelled material to be converted to the higher mol. wt. form. The processing of HA in BV1-MTHA/C127 cells (panel B) is even slower – even after a chase of 4 h, ~30% of the intracellular HA remains in a core-glycosylated state.

We have shown previously (Gething and Sambrook, 1982a) that the secreted form of HA (HA^{sec}) is processed more slowly in simian cells than the membrane-bound form. The same phenomenon occurs in murine cells (Figure 5, panel C). No labelled HA is secreted into the medium by BV1-MTHA^{sec}/C127 cells during the 30 min following a 15 min pulse of [^{35}S]methionine. Even after a 4 h chase with non-radioactive methionine, ~30% of the labelled HA remains associated with the cell in a core-glycosylated state. Little terminally-glycosylated HA is detected in the cell and conversely, no core-glycosylated HA is secreted from the cell. These result indicate that HA^{sec} travels very slowly from its site of synthesis in the rough endoplasmic reticulum to the Golgi apparatus. However, once terminal glycosylation has occurred, the molecule moves quickly to the surface of the cell where it is secreted into the medium without further delay.

Localization of HA by indirect immunofluorescence

We carried out indirect immunofluorescent labeling of the cell lines that produce HA to determine the location of the protein (Figure 6). When cells expressing the membrane-bound form of HA (69-MTHA/C127 or BV1-MTHA/NIH cells) were fixed and permeabilized before treating with anti-HA antibody (panels A and B), fluorescent staining was seen both at the cell surface, (reflecting the steady-state accumulation of HA in the plasma membrane), and intracellularly in the juxtanuclear region of the cell (presumably HA that was in transit through the Golgi apparatus). Cells expressing the truncated HA^{sec} displayed no diffuse surface fluorescence, but the perinuclear staining of the nascent HA in the endoplasmic reticulum and the Golgi apparatus could be seen clearly (results not shown). These patterns of fluorescence are similar to those previously reported for CV-1 cells infected with SV40-HA recombinant viruses (Gething and Sambrook, 1981, 1982b). Panel C shows the surface fluorescence observed on non-permeabilized BV1-MTHA/NIH cells. Virtually all cells of the population show specific staining, but the intensity of the fluorescence varies from cell to cell. This variation in intensity of surface staining can be quantitated more precisely by flow cytometry, and profiles of cell number *versus* log fluorescence are shown for 69-MTHA/C127 cells in panel D and BV1-MTHA/NIH cells in panel E. The surface fluorescence of 69-MTHA/C127 cells varies over a 30-fold range and the population of cells shows a unimodal distribution. The specific fluorescence of the cells at the lower end of the distribution curve is equal in intensity to the median level of background autofluorescence of the parental C127 cells: the medians of the two populations of cells differ by a factor of 10 in the intensity of their fluorescence: finally, 69-MTHA/C127 cells at the upper end of the distribution

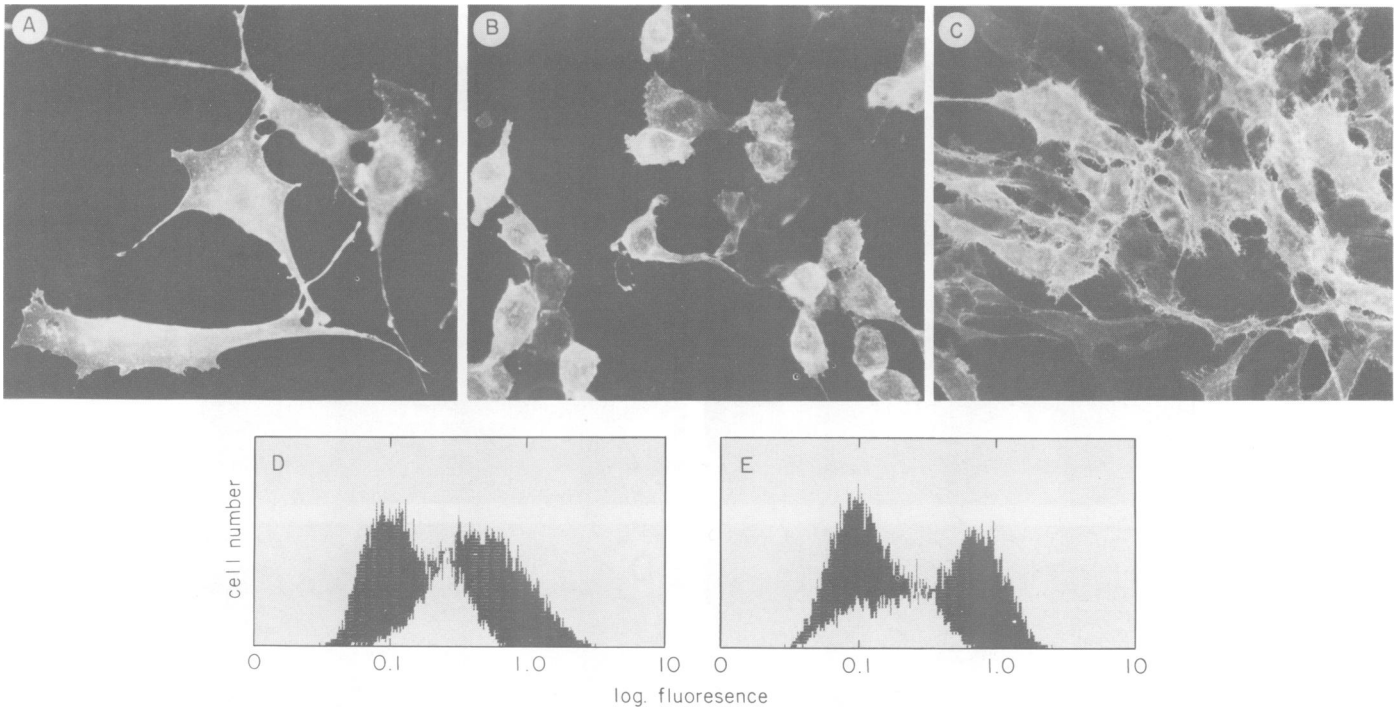


Fig. 6. Analysis of intracellular and surface HA on BPV-transformed cells by immunofluorescence and flow cytofluorometry. The conditions used for immunofluorescent staining of HA on fixed and permeabilized cells has been described previously (Doyle *et al.*, 1984). **Panel A,** permeabilized 69-MTHA/C127 cells; **panel B,** permeabilized BV1-MTHA/NIH cells; **panel C,** intact BV1-MTHA/NIH cells. The conditions used for cytofluorography on an Epics C fluorometer (Coulter) are described in Materials and methods. **Panel D,** 69-MTHA/C127 cells; **panel E,** BV1-MTHA/NIH cells.

curve fluoresce ~100 times more intensely than parental cells that display the median level of autofluorescence. The BV1-MTHA/NIH3T3 cells show a biphasic distribution of fluorescent intensity. In this experiment (panel E), ~30% of the cells fluoresced with the same intensity as the parental NIH3T3 cells: the majority of the population exhibited a level of specific fluorescence that was ~10-fold higher than the median level of autofluorescence. The reason for this cell-to-cell variation in fluorescent intensity is not yet known. However, it is unlikely to be due to genetic heterogeneity in the transformed cell populations since each of the cell lines shown in Figure 6 has been cloned and recloned at least twice. Several lines of evidence indicate that the pattern of distribution of fluorescence may be affected by the physiological state of the cells. For example, the relative size of the two populations of BV1-MTHA/NIH cells varies from experiment to experiment in a way that seems to depend at least in part on the state of growth of the cells. In some experiments only a few percent of the transformed cells fluoresce with the same low intensity as the parental cells; in other experiments such as that shown in panel E as many as 30% of the transformed cells show this low level of fluorescence. The heterogeneity may result from variation in the copy number of the BPV-HA vector or variation in the expression of HA as the cell passes through different phases of the division cycle. While further work is required to elucidate this problem, preliminary experiments in which the most fluorescent cells in the population were selected using a cell sorter suggest that it is feasible to obtain populations of cells that show, at least transiently, a more uniform and higher level of expression of HA. It is interesting to note that a similar heterogeneity of expression has been seen in populations of transformed cells that are expressing other proteins from BPV-derived vectors. For example, cloned lines of BPV-transformed murine C127 cells that express vesicular stomatitis G protein show at least 30-fold variation from cell to cell in the level of membrane-bound or secreted G protein (Florkiewicz *et al.*, 1983).

Detection of HA activity on the cell surface by binding of erythrocytes

To confirm that the membrane-bound form of HA is biologically active, we assayed the ability of the cell lines to bind guinea pig erythrocytes to their surfaces. The erythrocytes bound very inefficiently to cells that we knew from the previous experiments to be expressing considerable quantities of mature HA on their surfaces. Typically, only occasional cells (<5%) in cultures of cell lines such as BV1-MTHA/NIH or 69-MTHA/C127 (which on average express $>10^6$ HA molecules per cell) showed an ability to adsorb red blood cells (see Figure 7, panels A and C). Furthermore, when individual hemadsorbing cells were cloned and expanded into mass culture, the resulting sublines showed no greater ability to adsorb erythrocytes than the original line. These problems were resolved by treating the cultures with low concentrations of trypsin, neuraminidase, or chymotrypsin – enzymes that (particularly in the case of chymotrypsin) do not significantly affect the structure of native HA. Figure 7, (panels B and D) show the results of haemadsorbtion experiments carried out after confluent monolayers (siblings of the cultures shown in panels A and C) were treated with trypsin (2 μg/ml for 5 min at room temperature). The number of cells capable of haemadsorbing increased from <5% to >50% after treatment. With some cell lines (for example, BV1-MTHA/NIH, Figure 3B), it was possible to obtain populations in which >90% of the cells were capable of binding erythrocytes. There are at least two explanations of these results. The

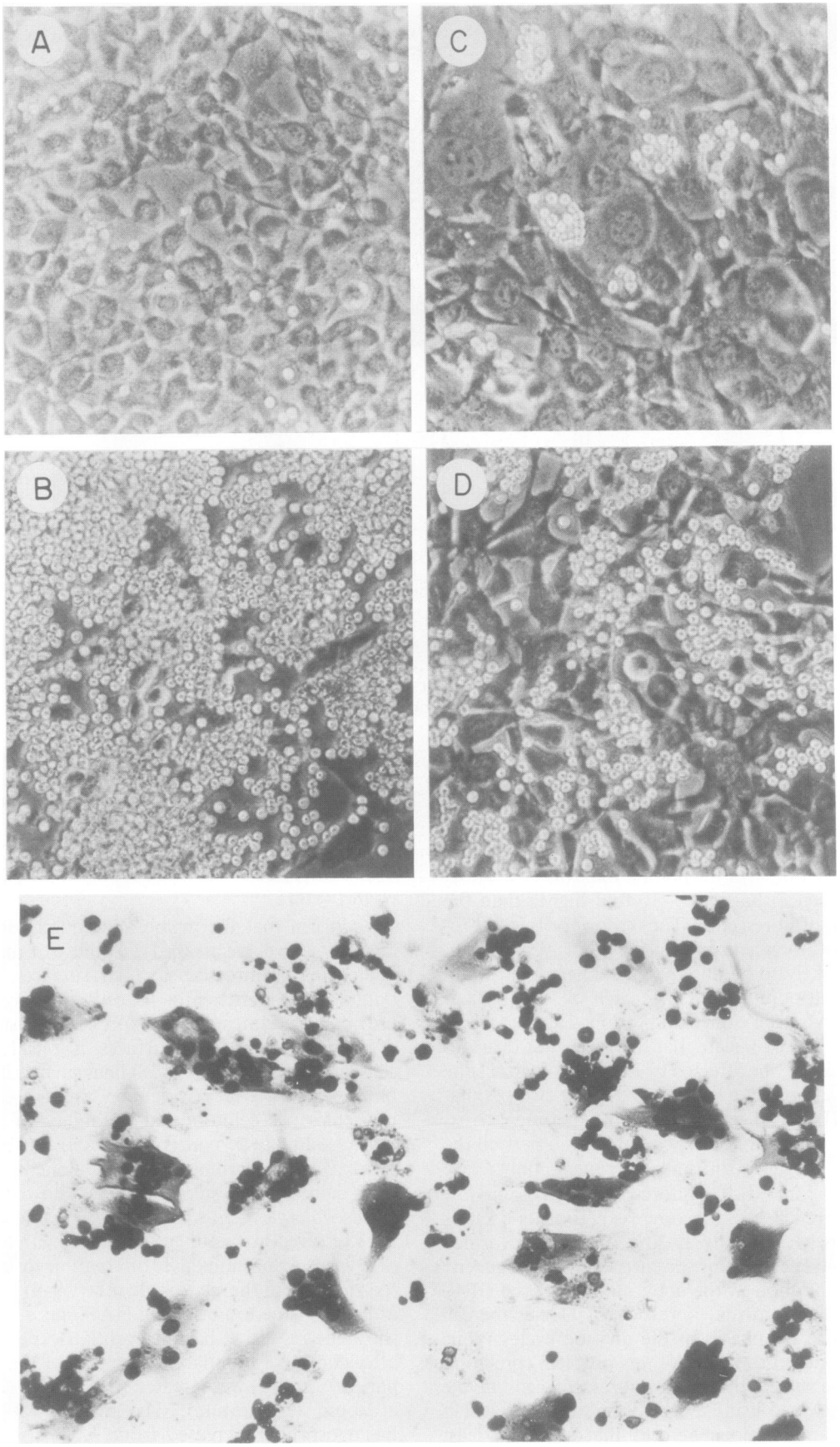


Fig. 7. Analysis of the erythrocyte binding and cell fusion activities of BPV-HA transformed murine cells. Monolayers of cells (**panels A and B,** BV1-MTHA/NIH cells; **panels C** and **D,** 69-MTHA/C127 cells) were incubated with guinea pig erythrocytes (1% solution in PBS) for 10 min at room temperature, washed thoroughly with PBS and photographed. In **panels B** and **D** the monolayers were treated with trypsin (2 μg/ml for 10 min at room temperature) before the red blood cells were added. **Panel E:** delivery of HRP into BV1-NTHA/NIH cell. The conditions used are described in Materials and methods.

simpler is that treatment with the enzymes removes an extracellular matrix that sterically or by its charge shields the HA molecules embedded in the cell surface from contact with erythrocytes in solution. This explanation is consistent with the fact that enzymes with very different specificities achieve the same effect. However, treatment with these enzymes could also alter the distribution of HA on the cell surface in a way that allows erythrocytes to bind more tightly. While we cannot exclude this explanation, we were unable to detect any change in the amount or distribution of HA (for example clumping or capping) when the surface of cells that had been treated with enzymes was examined by indirect immunofluorescence using anti-HA sera.

Not all cell lines were capable of binding erythrocytes, even after treatment with enzymes as described above. For example no hemadsorbtion could be detected to cell lines of the 69-SVHA/C127 or BV1-MTHA/MME series which produce only small amount of the membrane-bound form of HA, nor to any cell line that expressed the secreted version (HA^{sec}) of the HA protein. We believe that the concentration of HA on the surface of these cells is too low to allow binding of erythrocytes.

Fusion of BPV-HA transformed cell lines

The HA expressed in CV-1 cells from an SV40-HA vector is capable of inducing cell-cell fusion, provided that the protein is first cleaved by trypsin into HA1 and HA2 subunits (White *et al.*, 1982). The erythrocyte binding and membrane fusion activities of HA can be combined to effect the fusion of erythrocytes to CV-1 cells and to deliver the contents of red cell ghosts into the CV-1 cytoplasm (Doxsey *et al.*, in preparation). To determine if red cells could be fused to the cells which constitutively express HA from the BPV-HA vectors, erythrocyte ghosts were loaded with horseradish peroxidase (HRP) and allowed to bind to BV1-MTHA/NIH cells that had been treated with trypsin. Excess red cells were removed and the monolayer was incubated for a short period in medium at pH 5.0. After 45 min in normal medium, the cells were washed, fixed with glutaraldehyde and stained with diaminobenzidine to localize the HRP reaction product. Figure 7E shows that loaded erythrocyte ghosts can bind to BV1-MTHA/NIH monolayers, fuse with the cells after low-pH treatment and deliver the HRP into the cytoplasm. In such experiments HRP was delivered to ~25% of the cultured cells. Occasionally polykaryons could be observed, indicating that cell-cell fusion had occurred. Recently, the efficiency of delivery has been increased significantly in a derivative of the BV1-MTHA/NIH cell line selected for its high efficiency in binding red blood cells.

Future uses of these cell lines

When we began this work, we could see several obvious uses for cell lines that expressed large quantities of HA on their surfaces. They could be used in both immunological and biochemical experiments to investigate the interactions between a well-characterized foreign antigen and cellular surface components – for example, those coded by the major histocompatibility locus: the unique ability of HA to bind to erythrocytes and to fuse with membranes might allow the cells to serve as universal recipients for any materials of biological interest that could be loaded into red cell ghosts: and, because they express from multiple gene copies a surface protein that is not essential for the survival of the cell, they might be useful for the isolation of mutants of cellular genes – analogous to the *sec* mutants of yeast – that are defective at various stages of the secretory pathway. The first and second of these aims are now clearly feasible and the third still shows promise. The major question to be resolved is the cause of the heterogeneity of HA expression from BPV-derived vectors in cloned cell lines. If ways can be found to grow or select populations of cells that display uniformly high quantities of HA on their surfaces, the isolation of mutants that are unable to transport HA along the secretory pathway should become a fairly straightforward task.

Materials and methods

General methods

Preparation of plasmid DNA, digestion with restriction enzymes, agarose gel electrophoresis of DNA, transfer of DNA or RNA to nitrocellulose and hybridization, radiolabelling of DNA by nick-translation, end-labelling and repair of DNA, ligation, addition of synthetic linkers and bacterial transformation were done by standard methods (Maniatis *et al.*, 1982, and references therein).

Construction and properties of plasmids containing recombinant BPV-HA vectors

The plasmids used in this work were as follows.

(i) pBPV-BV1 (Zinn *et al.*, 1983), which contains the 5.5-kb subgenomic transforming fragment of BPV DNA (Lowy *et al.*, 1980), a deleted version of a 7.6-kb fragment of DNA derived originally from the human β-globin gene cluster (Bernards *et al.*, 1979; Fritsch *et al.*, 1980) and the plasmid pBRd (DiMaio *et al.*, 1982). This plasmid efficiently transforms murine C127 cells to yield cell lines that contain predominantly extrachromosomal copies of the transforming DNA (Zinn *et al.*, 1983).

(ii) pBPV69X, which contains the 5.5-kb subgenomic transforming fragment of BPV cloned into the *Xho*I site of pPX. The 5.5-kb fragment was excised from plasmid pBPV-H11 (DiMaio *et al.*, 1982) by cleavage with *Hind*III and *Bam*HI and purified by gel electrophoresis. The recessed 3′ ends of the linear DNA were then filled and ligated to synthetic *Xho*I linkers (Collaborative Research). After digestion with *Xho*I, the DNA was ligated to plasmid pPX that had been digested with *Xho*I and treated with alkaline phosphatase.

(iii) pPX, which consists of the *Cla*I-*Bam*HI fragment of plasmid Xf3 (Maniatis *et al.*, 1982) joined to a 2.8-kb segment of SV40 DNA extending counterclockwise from the *Hpa*II site at nucleotide 346 through the origin, early promoter and early coding region to the *Bam*HI site at nucleotide 2533. The *Hpa*II/*Cla*I site was then altered to an *Xho*I site by addition of synthetic linkers.

(iv) pJYM-MMTIa (Hamer and Walling, 1982), which consists of a 4.0-kb fragment of DNA containing the murine metallothionein gene cloned in plasmid pJYM. A derivative of pJYM-MMTIa, containing only the promoter region of the murine metallothionein gene was contructed by cleaving pJYM-MMTIa with *Bgl*II. The recessed 3′ ends of the linear DNA were filled and ligated to *Hind*III linkers (Collaborative Research). The DNA was then digested with a mixture of *Kpn*I and *Hind*III and the 600-bp fragment containing the promoter was isolated and subcloned into the 3.0-kb *Kpn*I-*Hind*III fragment of plasmid pPX. A plasmid (pMTH3) was isolated in which the *Bgl*II site at the distal end of the murine metallothionein promoter had been replaced by a *Hind*III site.

(v) pSVHA and $pSVHA^{sec}$ are two plasmids that consist of the 3.5-kb *Hind*III-*Bcl*I fragment of pPX and the coding region derived from a full-length cDNA clone of the hemagglutinin gene of the A/Jap/305/57 H2N2 strain of human influenza virus (Gething *et al.*, 1980) in either its wild-type or secreted (anchor-minus) versions (Gething and Sambrook, 1982). In these plasmids the hemagglutinin gene is placed between the SV40 enhancer-early promoter region and the polyadenylation site that marks the distal end of the SV40 early coding region (Gething and Sambrook, 1981).

pMTHA and $pMTHA^{sec}$ are plasmids of analogous structure except that the 366-bp *Kpn*I-*Hind*III fragment which spans the SV40 early promoter has been replaced by the 400-bp *Kpn*I-*Hind*III fragment of pMTH3 which contains the murine metallothionein promoter.

(vi) p69SVHA and $p69SVHA^{sec}$ (Figure 1a and b) are two plasmids that consist of the 5.5-kb subgenomic transforming fragment of BPV and the composite HA gene (promoter/coding region/polyadenylation site) from pSVHA or $pSVHA^{sec}$ cloned in plasmid pXf3.

p69MTHA (Figure 1c) is a plasmid of analogous structure, except that the composite HA gene (derived from plasmid pMTHA) contains the murine metallothionein promoter instead of the SV40 early promoter.

pBV1MTHA and pBV1MTHA^{sec} (Figure 1d and e) consist of the 5.5-kb subgenomic transforming fragment of BPV and β-globin DNA sequences (both derived from pBPV-BV1), and a composite HA gene (derived from pMTHA or pMTHA^{sec}) cloned in plasmid pBRd.

(vii) pON$_3$ (Hanahan, unpublished) contains a composite gene composed of the SV40 early promoter and the aminoglycoside phosphotransferase gene of Tn5.

Cells

Three murine cell lines, C127 (Lowy *et al.*, 1978), NIH3T3 and MME [a continuous line of polarized epithelial cells subcloned from MMTE cells (Damsky *et al.*, 1981)] were maintained in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum (Reheis) in a humidified atmosphere containing 5% Co_2 at 37°C. The cells were subcultured twice a week using 0.012% trypsin and were not allowed to grow to >40–60% confluency. Morphologically transformed clones derived from C127 cells were maintained in an identical fashion. Transfected derivatives of NIH3T3 cells or MME cells were initially selected for resistance to G418 but the cloned lines were subsequently maintained in medium lacking this antibiotic.

Transfection of cells with DNA

DNAs were introduced into the various cell types as a co-precipitate with calcium phosphate (Graham and van der Eb, 1973). BPV-HA plasmids (Figure 2) were introduced into C127, NIH3T3 or MME cells essentially as described by DiMaio *et al.*, (1982). The mixture used to transfect cells contained 1 μg of the appropriate plasmid and 20 μg of sheared carrier DNA from C127 cells. After exposure to the precipitate for 8 h, the cells were treated with 20% DMSO in complete medium for 3 min at room temperature. Foci of morphologically-transformed C127 cells were apparent after 10–14 days of incubation at 37°C. When resistance to the neomycin analog G418 (Jimenez and Davies, 1980; Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982) was used as a selectable marker, the transformation mixtures also contained 100 ng of plasmid pON$_3$. 24 h after co-transfection, the cultures were subdivided (split ratio 1:5) into medium containing G418 (0.3 mg/ml in the case of NIH3T3 cells; 0.6 mg/ml in the case of MME cells). Cell death usually began 3 days later and surviving colonies were apparent after 10–14 days of incubation. Colonies were picked using glass cloning cylinders into 12-well multiplates (Linbro). Clones selected for growth in G418 were assumed to have arisen from single cells and were not subcloned.

Preparation of cellular DNA

High mol. wt. DNA was extracted from cells as described by Wigler *et al.* (1979). Low mol. wt. DNA was extracted according to Hirt's procedure (1967), purified by two sequential extractions with phenol and concentrated by precipitation with ethanol.

Bacterial transformation

Transformation of bacteria (strain DH-1) was carried out according to Hanahan (1983). Routinely, transformation with 1 ng of pBR322 DNA yielded 3–8 x 10^4 ampicillin-resistant colonies.

Analysis of hemagglutinin

The methods for pulse-labelling of cells with [^{35}S]methionine in the presence and absence of tunicamycin, immunoprecipitation of radiolabelled hemagglutinin, analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis, quantitative radioimmune assay of HA and localization of HA within the cell by immunofluorescence have been described previously (Gething and Sambrook, 1981, 1982a).

Flow cytofluorometry

Cells were examined for expression of HA on the surface by quantitative cytofluorometry using an Epics C flow cytometer (Coulter). Cell lines were reseeded on polymethylpentene plates and incubated for 15–17 h at 37°C. Cells were then removed from the dish and incubated with a 1:100 dilution of a high titer rabbit antibody specific for the A/Japan/305/57 HA (Gething and Sambrook, 1981) for 30 min at 0°C. After repeated washings, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Cappel Laboratories) and incubated again at 0°C for 30 min. After further washing, the cell suspensions were examined by quantitative cytofluorometry.

Delivery of HRP into cells by HA-mediated cell fusion

Recipient cells were plated 48 h in advance onto 18 mm glass coverslips in 12-well Costar trays and grown to 80% confluency. They were then treated with trypsin (5 μg/ml in DMEM, no serum) for 10 min at room temperature and then with soybean trypsin inhibitor (10 μg/ml in PBS) for 5 min. After washing the cells twice in PBS, 0.5 ml of a 1% solution of HRP-loaded human red blood cells were added and let bind for 5 min at room temperature. Excess red blood cells were aspirated and the cells incubated in 2 ml fusion medium (PBS, 10 mM Mes, 10 mM Hepes; pH 5.0) for 1 min at 37°C. This solution was then removed and the cells incubated for 45 min in complete medium in a 37°C CO_2 incubator. At this time the cells were washed twice in PBS, fixed in 2.5% glutaraldehyde in PBS and stained with diaminobenzidine to localize the HRP reaction product. The details of this method will be published elsewhere (Doxsey *et al.*, in preparation).

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