Construction and packaging of pseudotype retrovirus containing human N-*ras* cDNA antisense sequence and its biological effects on human hepatoma cells

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ABSTRACT

N-*ras* is one of the transforming genes in human hepatic cancer cells. It has been found that N-*ras* was overexpressed at the mRNA and probein level in hepatoma cells. In order to explore the biological roles of N-*ras* in human hepatic carcinogenesis and the potential application in control of cancer cel1 growth, a pseudotype retrovirus containing antisense sequence of human N-*ras* was constructed and packaged. A recombinant retrovirus vector containing antisense or sense sequences of N-*ras* cDNA was constructed by pZIP-NeoSV (X) 1. The pseudotype virus was packaged and rescued by transfection and infection in PA$_{317}$ and ψ2 helper cells. It has been demonstrated that the pseudotype retrovirus containing antisense N-*ras* sequence did inhibit the growth of human PLC/PRF/5 hepatoma cells accompanied with inhibition of p21 expression, while the retrovirus containing sense sequence had none. The pseudotype virus had no effect on human diploid fibroblasts.

Key words: *N-ras cDNA, antisense RNA, retroviral vector, hepatic cell.*

INTRODUCTION

Protooncogenes and antioncogenes are believed to have regulatory roles in normal cellular proliferation and differentiation but also contribute to neoplastic transformation in their mutant forms with point mutation, rearrangement, amplification, deletion, transiocation or promoter insertion [1−3].

If mutant protoonoogenes have roles for abnormal cellular proliferation, then inhibition of their expression should alter cell growth. Specific antibodies may be used to block the action of oncogene products, and mioroinjeotion of antibodies directed against

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c-*ras* gene product has been shown to block the mitogenio response of NIH/3T3 cells to serum [4]. However, antibodies have limited value in analyzing the function of
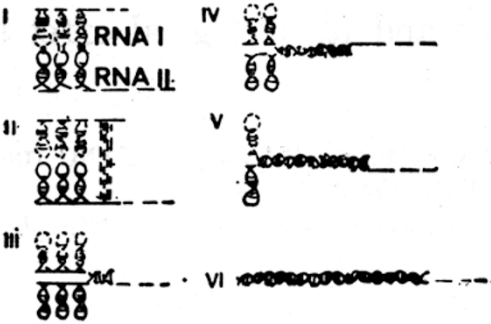

Fig. 1 Model for the stepwise binding of RNAI to RNAII. In step I RNAI and RNAII interact at the loops of their folded structure. Interaction facilitates pairing (step II) that starts at the 5' end of RNAI(step III). At this stage, the loop-to-loop contacts may be broken. Progressive pairing continues as the stem-and-loop structures unfold (steps IV and V). It should be noted that the three pairs of loops do not necessarily interact simultaneously, and RNAII may interact in an alternative folded structure. This figure was adapted from reference [6].

intracellular proteins. Antisense RNA has been proposed as a useful tool for inhibiting the function of specific genes [5]. RNA molecules containing sequences complementary to portion or complete RNA transcribed from a specific gene have been used to decrease the concentration of its gene product [6]. Inhibition results apparently from formation of RNA-RNA duplexes between the antisense RNA and RNA transcribed from the target gene (Fig. 1). Such RNA-RNA duplexes have been shown to inhibit mRNA translation but the intranuelear location of duplexes suggests that RNA processing or transport might be affected by antisense RNA [7]. Some results showed that antisense RNA could also be used to regulate oncogene expression [8−9]. However, the effect of antisense RNA was usually transient. The results from transfection with vectors containing antisense sequence were all transient and required drug selection due to low efficiency of DNA transfeetion.

Retrovirus are able to transfer their genetic information at high efficiency into eukaryotic cells. These viruses can be genetically manipulated by replacing their own genes with exogenous genes and thereby become vectors for gene insertion. Theoretically, up to 100 percent of cells can be infected and the integrated viral (and exogenous) genes can be expressed. The major utility of retroviruses is the availability of virus packaging cell line [10−11] which allows production of replication-defective retrovirus vectors in the absence of helper virus. Such vectors infect and integrate into cells but cannot replicate and spread. The absence of helper virus prevents the occurrence of new integration events. An additional important future usage of retrovirus may be in human gene therapy [12]. The authors reported here the use of a re~rovirus vector containing the oncogene antisense sequences for studying inhibition of target oncogene expression in human hepatoma cells.

N-*ras* is one of the transforming genes of human hepatic cancer cells. It has been found that in hepatoma cells, N-*ras* was overexpressed at the mRNA and protein level [13]. In order to explore the biological roles of N-*ras* in human hepatic carcinogenesis and its potential application for control of cancer cell growth, a pseudotype retrovirus containing antisense sequence of human N-*ras* was constructed and packaged. A recombinant retrovirus vector containing an antisense or sense sequences of N-*ras* eDNA was constructed by pZIP-NeoSV (X)1. The pseudotype virus was packaged and rescued by transfeetion and infection in PA_{317} and $\psi 2$ helper cells. It has been demonstrated here, that the pseudotype virus containing antisense N-*ras* sequence did inhibit the growth of human PLC/PRF/5 hepatoma cells accompanied with inhibition of p21 expression; while the virus containing

sense sequence had no inhibitory effect on hepatoma cells. The pseudotype virus had no effect on growth of human diploid fibroblasts.

MATERIALS AND METHODS

1. Plasmid construction and analysis of DNAs

The 1 kb N-*ras* cDNA sequence (contains the 5' portion of full-length N-*ras* cDNA) (2.3 kb)) and retrovirus expression vector pZIP-NeoSV(X)1 were obtained from Dr. Channing J. Der. Plasmid construction, DNA transformation, Southern blotting procedure and agarose gel electrophoresis were performed according to standard procedures [14]. Enzymes were obtained from Boehringer-Mannheinm Biochemica and were used according to manufacture's specification.

2. Cell culture, DNA transfection and viral infection

All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Plasmid DNA was introduced into PA$_{317}$ amphotropic packaging cells by means of calcium phosphate-mediated DNA transfer [15]. Twenty-four hours after 10^5 cells were seeded on T$-$25 flask (Corning), the cultures were transfeeted with 15 μg of plasmid DNA. In cases where selection for transfected cells was applied, cells were split 1:2 into selective medium containing 400 μg/ml of G418 24 hours after transfection. The medium was changed every 3 days and G418 resisbant foci were counted after 11$-$14 days. Permanently transfected PA$_{317}$ G418 clones were isolated after 10 to 14 days of selection, and expanded individually to confluence. The medium was replaced by G418 free medium and ~hen removed 24 hours later. It was centrifuged at 3000 rpm for 10 min to remove floating cells and debris. The supernatant containing viral particles could be used for transfection. The ecotropic packaging cell ψ2 were seeded at 3×10^5/T25 flask on day 1 On day 2 the medium was changed to medium containing 20 μg/ml of Polybrene and 2 ml of infectious virus supernantant were added, fresh medium was added 2 hours later. The medium was replaced with selective medium (400 μg/ml of G418). The medium was changed every 3 days and the G418-resistant foci were counted on the 14th days. By using the same procedure for amphotropic packaging cell PA$_{317}$, more amphotropic pseudotype viruses can be obtained.

3. Electron microscopy

Having been washed in 0.1M phosphate buffer (pH 7.4) at T25 culture flask, the transfected or infected cells were collected by centrifugation at 1500 rpm for 10 min (Sorvall SE-12 rotor). The cell pellet was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 hour at 4℃, post-fixed in 1% osmium tetroxide for 2 hours at 4℃, dehydrated in a series of alcohol and acetone, and embedded in domestics epoxy 618. Ultrathin sections were cut on LKB2188 Ultrotome Nova, stained by both uranyl acetate and lead citrate and examined under JEM-1200 EX electron microscope.

4. Infection of human hepatoma cells and human lung diploid fibroblast

Human hepatoma cell line PLC/PRF/5 was obtained from Professor Wen Yu-mei

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(Shanghai Medical University), Human lung diploid fibroblast obtained from Dr. He Meng-dong (Shanghai Sanitarian and Epidemiological Station). Infection procedure was same as the method described above. Cells were cultured on a small glass plate inside the dish in G418 free medium after infection. The glass plates were taken out in time for checking p21 expression by immune heroical method (ABC). At the same time the cells were also counted.

RESULTS

I. *Construction of retrovirus vector*

The 1 kb N-*ras* eDNA sequence was inserted into the BamHI site of retrovirus expression vector pZIP-NeoSV(X)I [16] (Fig. 2). The vector backbone consists of a Moloney murine leukemia virus (M-MuLV) transcriptional unit derived from an integrated M-MuLV provirus and pBR322 sequences necessary for the propagation of the vector DNA in *E. coli*. The specific M-MuLV sequences retained in the vector include the long terminal repeats (LTRs) necessary for the initiation of viral transcription and polyadenylation of viral transcripts as well as for integration, sequences for the reverse transcription of the viral genome, sequences for the encapsidation of viral RNA, and 5' and 3' splicing signals involved in the generation of subgenomic viral *env* RNA. In place of the retroviral sequences encoding the gag-pol polypeptide, a unique BamHI restriction site was inserted, to permit the expression of sequences introduced into the vector from either the full-length or spliced retroviral transcripts [17]. DNA sequences derived from the TS, which encode G418 resistance in mammalian cells and kanamycin resistance in *E. coli*, as well as sequenees encoding the SV40 and pBR322
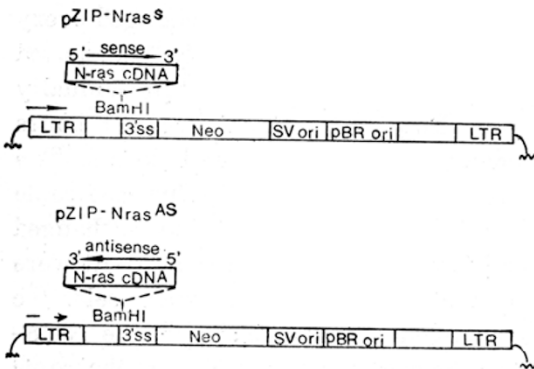

Fig. 2 Schematic structure of recombinant retroviral vectors containing human N-*ras* cDNA in sense or antisense orientation. Human N-*ras* cDNA fragment (1 kb) cloned into the BamHI site of retroviral vetor pZIP-NeoSV (X)1 (10 kb). Arrows demonstrated the orientation of the human-N-*ras* cDNA fragment are listed as 5' to 3' (sense, pZIIP-Nrass) oz 3' to 5'(antisense, pZIP-N*ras*AS). Other components of the vector include M-MuLV long terminal repeat (LTR), a 3' splice acceptor sequence utilized in the formation of subgenomic RNA 3' ss, the neomycin phosphotransferase coding sequence (Neo), a SV40 replication origin (SVori) and a pBR322 plasmid replication origin (pBRori).
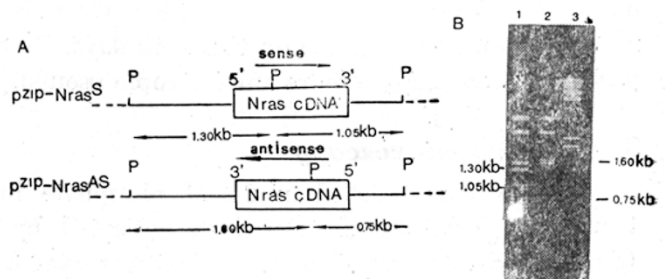

Fig. 3 A. Schematic structure of the 1kb N-*ras* eDNA insert on retroviral vector in sense or antisense orientation. Restriction site, P, PvuII.
B. Agarose gel electrophoresis of pZIP-N*ras*S and pZIP-N*ras*AS restriction digestiom.
1. pZIP-N*ras*S+PvuII
2. pZIP-N*ras*AS+PvuII
3. λ + HindIII

origins of replication have been introduced. These sequences allow for the selection of mammalian cells harboring the SVX provirus and for the rapid recovery of free or integrated proviral gonomes as bacterial clones. After ligabion between N-*ras* cDNA and pZIP-NeoSV(X) 1, the recombinants were isolated and analyzed (Fig. 3). There were two types of recombinants, one was the insert in the same orientation (sense) to the viral vector transcriptional unit from 5' to 3', termed as pZIP-Nras[S]. It could produce the transcript of N-*ras* cDNA inserk The other one was the insert in an opposite orientation to the viral vector transcriptional unit from 3' to 5', designated as pZIP-N*ras*[AS]. The viral genomic RNA complementary to the N-*ras* mRNA could act as an antisense N-*ras* RNA.

2. *Generation of pseudotype retrovirus packaging cell lines*

Helper-free virus was made from the vectors depicted in Fig. 2 by using PA317 retrovirus packaging cells. Virus produced by PA_{317} cells has an amphotropio host range that allows infection of cells from most mammalian species, including human [18]. To generate vector-producing cell line, the plasmid forms of vectors (pZIP-NeoSV (X) I, pZIP-N*ras*[S] and pZIP-N*ras*[AS]) were introduced in to PA_{317} respectively by calcium-phosphate-mediated transfeofion, and the stable G418-resistant foci were obtained in selective medium. It was found that the G418-resistant PA_{317} cells contained unrearranged proviruses in the cellular chromosomes by Soubhern blot analysis (data not shown). For infection, the viruses produced by PA_{317} were harvested and used to infect ecotropic retrovirus packaging cells $\psi 2$, and the G418-resistant foci were obtained. The second infection cycle consisted of $\psi 2$ produced pseudobype viruses for infecting PA_{317} cells. The culture supornatant from PA_{317} has a titer of $1\text{-}2\times10^3$ G418 cfu/ml on $\psi 2$ cells. Results showed that the virus packaging cell lines PA_{317} and $\psi 2$ could produce pseudotype retroviruses efficiently.

3. *Electron microscopic observation of the virus particles*

The retrovirus particles were found both in transfected PA_{317} cells and infected cells (including PA_{317} and $\psi 2$).

Two forms of particles were shown under electron microscope: the first one was $80-100$ nm in diameter, had an undense core with two layers of membrane, spikes were projecting on the surface. These particles were scattered in the cytoplasm, sometimes with budding from rough endoplasmic reticulum. The second form had an electron dense circular or horseshoe shaped core, dia, $70-80$nm, and displayed a typical structure of retrovirus. It located mainly in rough eudoplasmic reticulum and occasionally in perinuclear space (Fig. 4). By dot hybridization analysis of viral genemie RNA containing N-*ras* insert using N-*ras* probe, it was demonstratod that the culture supernatant from infected PA_{317} contained the recombinant pseudotype retrovirus (data not b shown).

4. *Biological effects of pseudetppe retroviruses containing Human N-ras sense or antisense sequences*

The experiments to observe the biological effect of pseudotype retrovirusos containing human N-*ras* sense or antisense sequence have been performed for three times. In all the

experiments, significant inhibitory effect has been observed on the growth of PLC/PRF/5 human hepatoma cells infected with virus containing antisense N-*ras* sequence as compared with those with sense sequence and those without N-*ras* insert. The representative data of the last experiment were demonstrated in Fig. 5A. The data of cell count of hepatoma cells per each time interval infected with different pseudotype viruses represented an average value of three bottles of cells. Since the growth of hepatoma cells after infected with retrovirus containing antisense N-*ras* sequence was inhibited, it was difficult to amplify the cells and to extract the protein. Therefore, the expression of N-*ras* product, p21 was observed by immunocytochemical assay using a monoclonal antibody against N-*ras* gene product prepared by Hong *et al*. [19]. The expression of p21 in PLC/pRE/5 cells was dramatically inhibited after infected with antisonso N-*ras* retrovirus (Fig. 6) as compared with cells infected with viruses containing sense sequence or those without insert. By using those recombinant psoudotype viruses, human lung diploid fibroblast was infected. Results showed that both pseudobypo virus, either containing sense or antisonso N-*ras* sequences had no effect on fibroblast (Fig. 5B). This may imply that antisense N-*ras* RNA produced by pZIP-N*ras* has a preferentially biological effect for nepatoma cells, in which the N-*ras* gone was over-expressed.
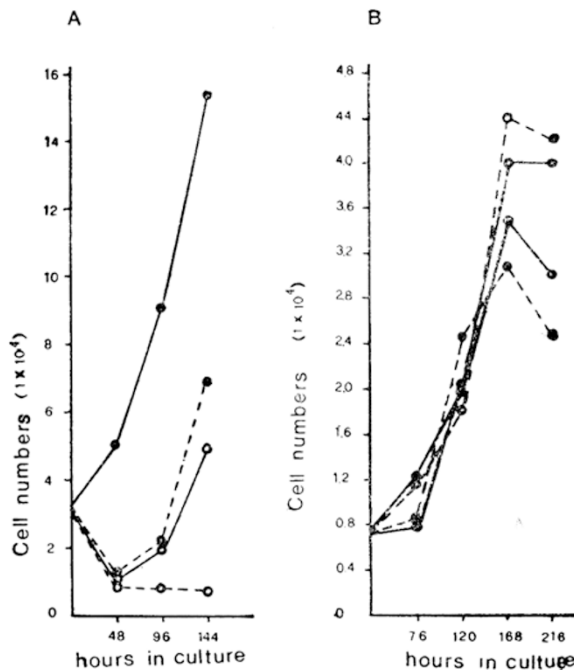

Fig. 5 Biological effect of recombinant psendotype retroviruses containing sense or antisense human N-*ras* cDNA on the growth of hepatoma cells PLC/PRE/5 or human diploid fibroblasts. The dose of pseudotype viruses to infect cells was at M. O. I of 1.0. A. Growth rate of PLC/PRF/5 infected with various retroviruses: ● − ● the control; ● − ● pZIP-NeoSV(X)1; ○ − ○ pZIP-N*ras*^{S}; ○ − ○ pZIP-N*ras*^{AS}. B. The growth rate of human diploid fibroblasts infected with various retroviruses.

DISCUSSION

From the data presented, it is evident that the rebrovirns vector containing an autisense N-*ras* sequence could inhibit the growth of human hepatoma cells but not the human diploid fibroblasts. It implicated that the inhibitory effect of cell growth is not attributed to the non-specific toxic effect of retrovirus introduced into cells. Furthermore, a single infection of antisense retrovirus construct could arrest the hepatoma cell to grow for 6 days, indicating much powerful than the effect of antisense oliogonucleotides. All these data implicated the potential value in future cancer gone therapy.

However, the mechanism of the preferential inhibitory effect of human hepatoma cells by the antisense N-*ras* sequences remains unclear. We can ask why the antisense sequence has no remarkably inhibitory effect on human fibroblast in which N-*ras* does express at a certain extent, though at a much lower level [20]. Several possible assumptions could be made to be further testified. First of all, the biological effect of antisense sequences to block oncogene/protooncogene expression might depend on the cell types as well as their physiological state. For hepatoma cells, N-*ras* is only one of the activated oncogenes which are essential for carcinogenesis or progression [21]. It seems likely that for the maintainance of malignant phenotype, cancer cells require the collaboration of a battery of activated oncogenes. Once the N-*ras* expression has been suppressed to a certain extent, the remaining oncogenes might be not suffieient to maintain the persistant proliferation, eventually leading to growth arrest. However, in case of normal diploid fibroblasts, the normal cell cycle could still keep process even if the expression of one proto-oncogone has been suppressed, possibly compensated by the other protooneogene(s) under some unknown cellular regulatory mechanism. Secondly, as predicted by Hirakawa and Ruloy [22], the sensitivity of tumor cells to treatment by suppression of some oncogenes might exceed that of normal cells, therefore favoring the strategic concern of gene therapy. Thirdly, the molecular mechanism of interaction between retrovirus carrying an exogenous gone fragment and host genome, and its messengers is not well elucidated. Therefore, experiments should be performed for further justification. Moreover, it is worthnoting that the cDNA insert in our retrovirus vector construct is only 1.0 kb, and therefore it can not be expected that the sense cDNA could give rise to a full length 2.3 kb N-*ras* mRNA. Recently it has been reported that *ras* gene with mutation at certain site could inhibit the growth cells transformed by v-*ras* (Liu Dinggan *et al*, to be published, 1989). Also, *ras* gone analogs such as K-*rey*-1 could reverse the cells transformed by Ki-*ras*[23]. Therefore, the biological activity of retrovirus containing incomplete N-*ras* cDNA sequence should be subjected to further study.

ACKNO WLEDGEMENT

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postgraduate thesis of Jia Li-bin and Wang Xiang.

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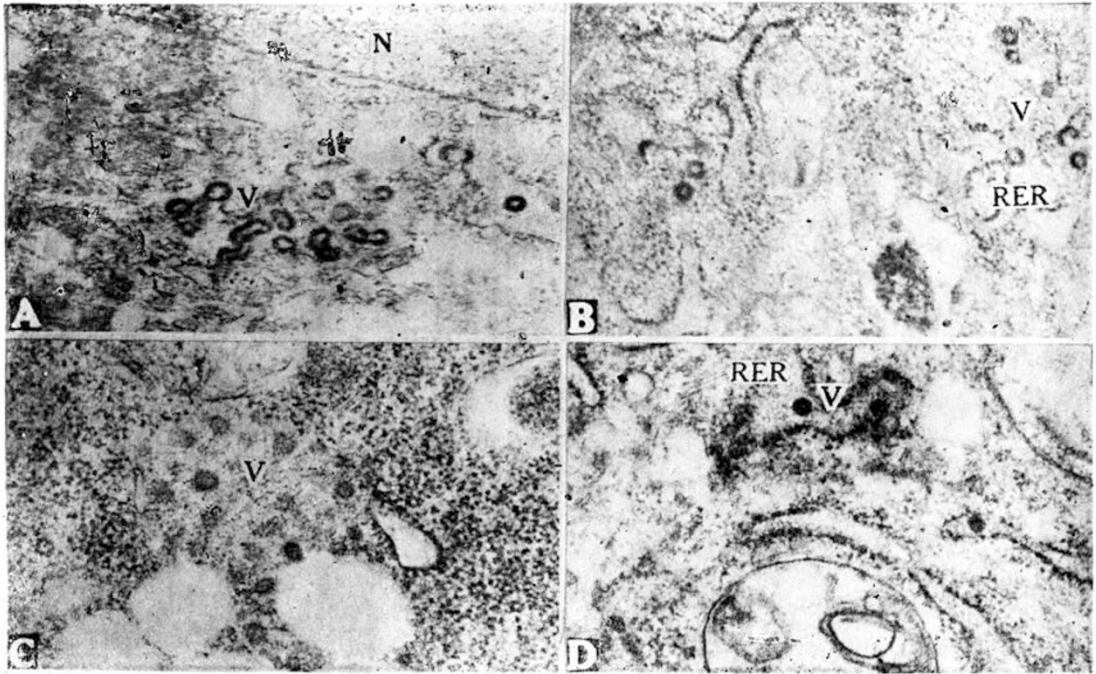
Fig. 4 Electron micrographs of pseudotype retroviral particles. A. ψ2 cells infected with pZIP-N*ras*^{S} retroviruses, ×40,000, the viruses (V) with circular structure ia clumps of cytoplasm. B. ψ, 2 cells infected with pZIP-N*ras*^{AS} retroviruses there were viruses (V) with circular or horseshoe core in rough endoplasmic reticulum (RER). C. PA$_{317}$ cells infected with pZIP-N*ras*^{S} retroviruses, virus particles (V) which had an undense core and spike projecting on the surface were found in cytoplasm. D. PA$_{317}$ cells infected with pZIP-N*ras*^{AS} retraviruses, virus particles with circular electron dense core were found in rough endoplasmic reticulum (RER).
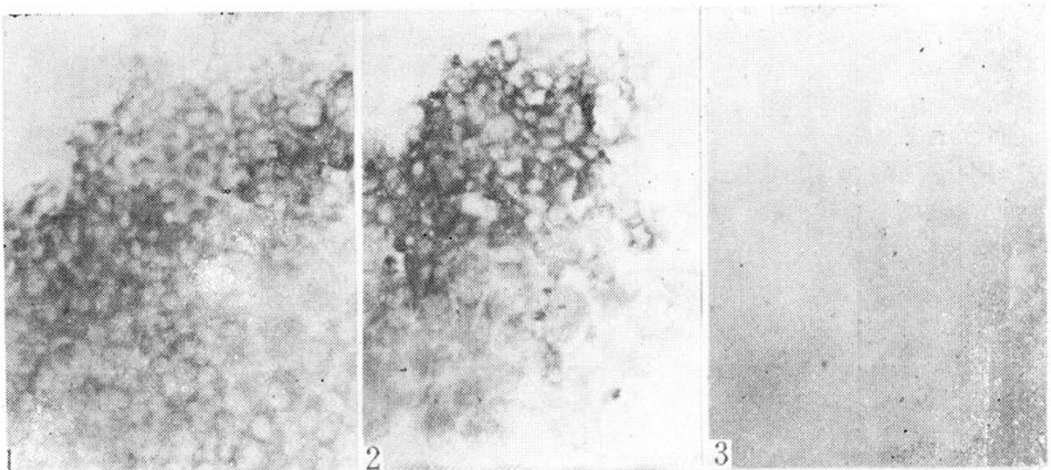

Fig. 6 P21 expression assay with peroxidase stained cells. PLC/PRF/5 cells infected with pseudotype retroviruses, and stained with moneclonal antibody for N-*ras* followed by an arlti-mouse IgG antibody conjugatd with peroxidase. 1. Cells infected with pZIP-NeoSV (X)1 (vector) retroviruses. 2. Cells infected with pZIP-N*ras*^{S} retroviruses. 3. Cells infected with pZIP-N*ras*^{AS} refroviruses.

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