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Regulation of Expression of a Retroviral Transforming Gene and Its Cellular Homolog

Bernard Mathey-Prevot

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Bernard Mathey-Prevot

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My sincere hope is that I will not fail the people I had the good fortune to work with. I had the rare priviledge to be surrounded by superb scientists, who not only taught me a scientific method, but also showed me that excellence and modesty need not be mutually exclusive. In that, I have a special debt to Masabumi Shibuya and Tatsuo Takeya, who on top of everything, proved to me that Japanese have a fine sense of humor. I would like also to acknowledge Ricardo Feldman for his patience, understanding and advice throughout these years. Special thanks go to Teena Lerner, Fred Cross and Ben Neel with whom I shared the fun and predicament of a graduate student life in the lab, and for their input in my work through stimulating discussions and advice.

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Summary

This thesis has focused on the avian Fujinami sarcoma virus (FSV) and its role in transformation. Specifically, it included the biochemical characterization of temperature sensitive (ts) mutants of FSV, the identification of the product of a cellular gene homologous to the transforming gene of FSV, and the isolation and characterization of phenotypic revertants of rat cells transformed by FSV.

Analysis of the defect of the two ts mutants of FSV revealed that P130 is the transforming protein of FSV, and that its kinase activity plays a crucial role in cell transformation by this virus.

v-*fps*, the transforming gene of FSV is homologous to a cellular gene, c-*fps*, present in uninfected cells. We obtained a specific antiserum directed against the v-*fps* domain of P130. This antiserum specifically crossreacts with a cellular protein of MW 98,000 daltons, present in uninfected chicken bone marrow cells. Like P130, this normal cellular protein (NCP98) is a phosphoprotein which is associated in vitro with a protein kinase activity specific for tyrosyl residues. In addition, tryptic peptides analysis showed that NCP98 is structurally related to P130. The expression of NCP98 is tissue specific, and highest in bone marrow cells of the granulocytic lineage.

The isolation of phenotypic revertants of rat cells transformed by FSV suggested a relationship between the expression of P130, its enzymatic activity and cellular transformation. Three types of revertants have been characterized, all

of them susceptible to retransformation after superinfection with FSV. Type I revertants (3 clones) have lost the complete FSV provirus. Type II revertant (1 clone) has a mutated FSV provirus which encodes a transformation defective protein of MW 130,000. This protein has no kinase activity in vitro, is underphosphorylated and lacks phosphotyrosine in vivo. In superinfected clones, it appears to be a substrate of P140, the transforming protein of another strain of FSV used in the superinfection. The mutated protein is associated in vivo with two cellular proteins (90K and 50K) which have been implicated in the binding of the transforming proteins coded by conditional mutants of avian sarcoma viruses. Type III revertants (11 clones) have the following properties: (1) The FSV provirus and integration site are identical to those of the parental clones. (2) No or very little v-fps mRNA is synthesized; likewise, P130 is not detected, or is present in very small amount. (3) Each revertant segregates retransformants with a frequency ranging from 10^{-6} to 10^{-7}. (4) There is an inverse correlation between the level of methylation of FSV proviral DNA and the level of expression of FSV mRNA. (5) DNase I hypersensitivity of the FSV provirus in revertants is abolished.

Finally, in clones of rat fibroblasts showing different degrees of transformation after FSV infection, we observed a good correlation between the amount of v-fps mRNA in a cell and its phenotype. Therefore, we propose that transformation by FSV is a function of the dosage of the v-fps gene, and not an all or none phenomenom.

List of Abbreviations

AEV	: avian erythroblastosis virus
ALV	: avian leukosis virus
AMV	: avian myeloblastosis virus
ATP	: adenosine triphosphate
BSA	: bovine serum albumin
B77	: Bratislava 77 strain avian sarcoma virus
C_rt	: molar concentration times time in seconds
$C_rt_{1/2}$	: molar concentration times time in seconds at which 50% hybridization is achieved.
cDNA	: complementary DNA
DTT	: dithiothreitol
EDTA	: ethylenediamine tetraacetic acid
FeSV	: feline sarcoma virus
FSV	: Fujinami sarcoma virus
GA-FeSV	: Gardner-Arnstein strain of FeSV
Ha-MuSV	: Harvey strain of MuSV
Ki-MuSV	: Kirsten strain of MuSV
LTR	: long terminal repeat
MuLV	: Murine leukemia virus
MuSV	: Murine sarcoma virus
NY225	: FSV mutant, temperature sensitive for transformation
NY240	: FSV mutant, temperature sensitive for transformation
RAV-2	: Rous-associated virus 2
RIPA	: radioimmunoprecipitation assay buffer
RSV	: Rous sarcoma virus
SDS	: sodium dodecyl sulfate
TBR	: tumor-bearing rabbit
td	: transformation defective
ts	: temperature sensitive

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INTRODUCTION

Although cancer is recognized as the uncontrolled growth of malignant cells, the exact mechanisms that trigger a cell to become cancerous still remain unclear.

RNA tumor viruses constitute an ideal system for studying these mechanisms. They generally do not kill the host cell, and are maintained essentially as cellular elements, integrated in the host chromosome. They contain a small number of genes that are expressed at high levels in infected cells, making the identification of their products convenient. But perhaps most intriguing is the ability of many of the retroviruses to transform cells in tissue culture and cause neoplastic disease in animals.

Taxonomy: the avian leukosis-virus group belongs to the Oncovirinae subfamily of the Retroviridae family [78]. This classification is based on the structure of the virus particle and their unique mode of replication (RNA -> DNA -> RNA) which provided the first exception to the central dogma that the flow of genetic information is unidirectional, from DNA to RNA.

Avian retroviruses can be further classified as either slowly transforming or rapidly transforming viruses, based on their pathological properties.

Avian Leukosis Viruses: avian leukosis viruses (ALVs) are the prototypes of the slowly transforming viruses. They contain three structural genes required for virus replication, namely gag (coding for the structural proteins or group specific an-

tigens), pol (coding for the reverse transcriptase) and env (coding for the envelope glycoproteins). Their genome consists of two chemically identical copies of capped, poly(A)-containing RNAs, which are held together at their 5'termini. This 60-70S complex is packaged with some structural proteins as a nucleoprotein complex inside a core particle, consisting of the mature gag-encoded proteins p19, p27, p12 and p15, which are derived from the precursor protein $Pr76^{gag}$. A number of low molecular weight (4-7S) cellular RNAs are found in the core particle, either free or associated with the 60-70S complex. Also within the core are 10-20 molecules of reverse transcriptase, the enzyme responsible for copying the RNA genome into the double stranded DNA . A fifth structural protein, p10 (also derived from $Pr76^{gag}$), has been recently detected and found to be between the core particle and the viral envelope [96]. Surrounding the core is the lipid envelope, derived from the host cell plasma membrane. Embedded in it and protruding as spikes are the virally encoded glycoproteins gp85 and gp37, linked together by disulfide bridges. These glycoproteins are responsible for the virus-host specificity of infection, and define seven viral subgroups (identified by the letters A through G). ALVs do not contain any transforming genes, but exert their neoplastic action by activating the expression of a cellular gene by a mechanism which was recently elucidated [54].

Life cycle: the first step in the life cycle begins with the non-specific attachment of the virus to the cell. It is fol-

lowed by penetration which involves the specific interaction between the viral glycoproteins and cellular receptors. In the cytoplasm, the reverse transcriptase copies the viral genomic RNA into cDNA. The initiation of DNA synthesis requires a primer molecule which, in the avian system, is a host derived $tRNA^{trp}$ [25]. The tRNA is base-paired to a region about 100 nucleotides from the 5' end of the genome, and DNA synthesis proceeds leftward to the 5' end of the template. It then "jumps" to the other end of the RNA template, by taking advantage of the terminal repeats, R, present at either end of the viral genome, and which provide a region of homology between the newly synthesized 100 nucleotides and the 3' end of the viral RNA. DNA synthesis then proceeds in the usual 5' to 3' fashion, again to the 5' end of the RNA to complete the minus strand DNA. Synthesis of the plus strand DNA probably initiates at a site located near the 3' end of the genome and proceeds rightward. In an analogous fashion to the minus strand synthesis, a second jump to the 5' end of the template is required to complete the plus strand synthesis. This particular mode of replication generates linear, double-stranded DNA molecules, which have redundant termini designated long terminal repeats (LTRs). LTRs contain the region U_3, the unique region derived from the 3' end of the viral RNA, the repeat R, and U_5, the unique sequence derived from the 5' end of genomic RNA; these sequences are arranged in the 5' to 3' order: 5'-U_3-R-U_5-3'. LTRs are important regulatory elements since they contain sequences involved in initiation of viral

transcription and poly(A) addition [129]. Linear duplexes appear a few hours after infection in the cytoplasm, and are transported to the nucleus, where some of them are converted to closed circular forms bearing one or two LTRs. The DNA then integrates into the host chromosome via a mechanism which is not completely understood. It is believed that closed circles (either with one or two LTRs) may be intermediates in integration. There are no preferred sites in the host DNA for integration , but this process involves a specific site in the viral sequence. Eventually, a double stranded DNA molecule, colinear with the viral genome and flanked by a set of LTRs at either end, is found covalently integrated in host cell DNA. Some 4-6 nucleotides of cellular DNA adjacent to the 5' LTR are directly repeated adjacent to the 3' LTR. The exact number of nucleotides that are repeated, varies in different virus species [129].

The provirus behaves essentially as a stable host cell genetic element. It is transmitted in an heritable fashion at each cell division (vertical transmission). It serves as template for viral RNA synthesis, and some of the genomic RNAs will be packaged into virus particles, which can infect new cells (horizontal transmission). In ALV-infected cells, two types of viral RNAs are made: one type is a 35S RNA, or genomic RNA, which can either be packaged in virions or can be used as a message for the two precursor proteins, $Pr180^{gag-pol}$ and $Pr76^{gag}$. These polyproteins are subsequently cleaved into the reverse transcriptase and the mature structural proteins,

respectively. The second RNA species is a 21S subgenomic RNA, which has the 5' leader sequence of the genomic viral RNA spliced to the env sequences. This RNA directs the synthesis of the precursor protein $Pr62^{env}$, which is cleaved posttranslationally, and processed to the gp85-gp37 glycoprotein complex.

Virion assembly takes place on the cell membrane, and viral particles bud off into the environment without killing the host cell. The released particles are then ready to start a new round of infection. Already infected cells, however, are resistant to superinfection by viruses of the same subgroup. This viral interference is caused by the blocking of cell receptors [104].

Rapidly transforming viruses: Unlike ALVs, this group of viruses induces a variety of neoplasms in infected animals after a short latency, and is capable of transforming cells in tissue culture. The first isolate, Rous sarcoma virus (RSV), was a sarcomagenic virus obtained by Rous from a chicken sarcoma [103]. RSV genome was shown to contain, in addition to the three structural genes of ALVs, extra sequences near the poly(A) end of the RNA. This difference between the genetic structure of RSV and ALVs firmly established the existence of a gene in RSV which was responsible for the sarcomagenic property of RSV, and this gene was called src [136].

At that stage, two seminal observations were made. The first dealt with the nature of the transforming src gene. Stehelin et al. successfully prepared cDNA that was specific for the viral src gene; when $cDNA_{src}$ was hybridized to DNA iso-

lated from uninfected chicken cells, the kinetics of reassociation of this probe to cellular DNA revealed that it contained one to two copies of related sequences [122]. This discovery made plausible the theory that viral src could have been derived from a cellular gene, c-src, as it was later called. The second observation confirmed that hypothesis. H. Hanafusa et al. injected young chicks with a mutant of RSV which was transformation defective (td virus) as a result of a large deletion that spanned almost the entire src gene. After a relatively long latency (two to three months), the birds developed tumors at sites distal to the site of injection, and rapidly transforming virus was recovered from the tumors [50]. These recovered transforming viruses were considered to have been generated by recombination between td virus and c-src sequences, and this was later confirmed by analyzing RNase-T1 resistant oligonucleotides of the src gene, present in the recovered viruses [137].

The type of analysis pioneered by Stehelin and others was extended to other rapidly transforming viruses. It allowed the characterization of the genetic structure of different transforming genes, and led to a classification of these viruses based on the relatedness of their transforming sequences [18]. In each case, a cellular gene was found to be homologous to a given viral transforming gene. These studies also revealed that, unlike in RSV, the transforming gene of most of these viruses replaced part or most of the replicative genes in the viral genome, with the consequence that such

viruses were deficient in replication. Based on these results, it was proposed that the rapidly transforming viruses had arisen by recombination between a helper-like virus and a unique cellular sequence (c-onc gene), which, once transduced by the virus (v-onc gene), showed strong transforming activity. Furthermore, such recombination had been made in most cases at the expense of the genes of the helper virus.

Several striking features make these c-onc genes intriguing: first, they have been shown to be present in organisms that are phylogenetically distant, and they exhibit a remarkable degree of conservation, even among distant species [118,120]. Second, these genes are expressed at very low levels, unlike their viral counterpart, which are expressed at high levels in infected cells. Finally, there is mounting evidence that some of these genes are differentially transcribed, showing a tissue specificity of expression, which in some cases, correlates with the stage of differentiation or development of an organism [3,83,108,112,115,142]. These features argue strongly for a central role that such genes might have in these processes at an early stage, common to many different species, since they are shared by evolutionary distant species.

Recently, additional cellular oncogenes have been identified, independently of their transduction by retroviruses, by assaying the ability of cellular DNA to transform mouse fibroblasts NIH 3T3 upon transfection (for review, see [139]).

c-onc gene products: Because of the very low expression of the c-onc genes and also of the lack of appropriate reagents that

could recognize their gene products, the study of c-onc proteins has lagged behind that of the viral proteins. Nevertheless, the first protein to be characterized was the product of the c-src gene, $p60^{c\text{-}src}$ [19,89]. It proved to be very similar in its structure and biochemical properties to the viral $p60^{v\text{-}src}$. One notable exception to this high degree of structural homology was found to reside in the C-terminal end, where the last twelve amino acids of $p60^{v\text{-}src}$ are replaced by nineteen amino acids in $p60^{c\text{-}src}$ [126]. Subsequently, three other cellular oncogene products were described. Two products, the c-abl and c-fes proteins, were shown to be immunologically related to the transforming proteins of Abelson murine leukemia and feline sarcoma virus respectively, whereas the third protein, the c-ras protein, was also characterized at the structural and biochemical levels [3,66,112,142]. The central question with these proteins is whether, under the appropriate conditions, they can transform the tissues in which they are expressed. In the case of c-ras, either high expression of the c-ras protein, $p21^{c\text{-}ras}$, or a single amino acid replacement in $p21^{c\text{-}ras}$, led to transformation [27,101,124]. Therefore, transformation by c-onc proteins might be achieved either through enhanced expression and/or a mutation of the protein, but the situation may vary with each cellular oncogene product, and more information about these proteins is needed in order to settle this point.

Fujinami sarcoma virus. Avian sarcoma viruses belong to the group of the rapidly transforming viruses, and their classifi-

cation has been based on the nature of the unique sequences which are responsible for their sarcomagenic properties. Four different genes have been identified, namely src, fps, yes, and ros [87,115]. Fujinami sarcoma virus (FSV) contains the fps gene, which is also present in PRCII, UR1, and 16L viruses [51,69,84,114,138]. The FSV genome (Fig. 1) lacks pol, env and part of gag, but contains instead the newly acquired, fps sequence [51,69]. Because of the substantial substitution of the replicative genes, FSV replicates only in the presence of a helper, of the ALV type, leading to FSV pseudotyped particles which are infectious. When a regular stock of virus, containing both FSV and helper particles, is used at an extremely low multiplicity of infection, some cells will be only infected by an FSV pseudotyped particle. These cells are transformed, but do not produce any virus particles.

These cells synthesize a 28S viral RNA (genomic RNA), which is translated into a single product, P140, a transforming protein of 140,000 daltons [51,69]. There are two strains of Fujinami sarcoma virus, one coding for the P140 protein, and the other encoding a slightly smaller protein, P130, but otherwise identical to P140. P140 or P130 will be used interchangeably in this text, in accord with the strains used in published experiments or in this study.

P140 is a polyprotein containing gag determinants at its N-terminus fused to the unique portion derived from v-fps (Fig. 1). It is a phosphoprotein in vivo, which contains both phosphoserine and phosphotyrosine [35].

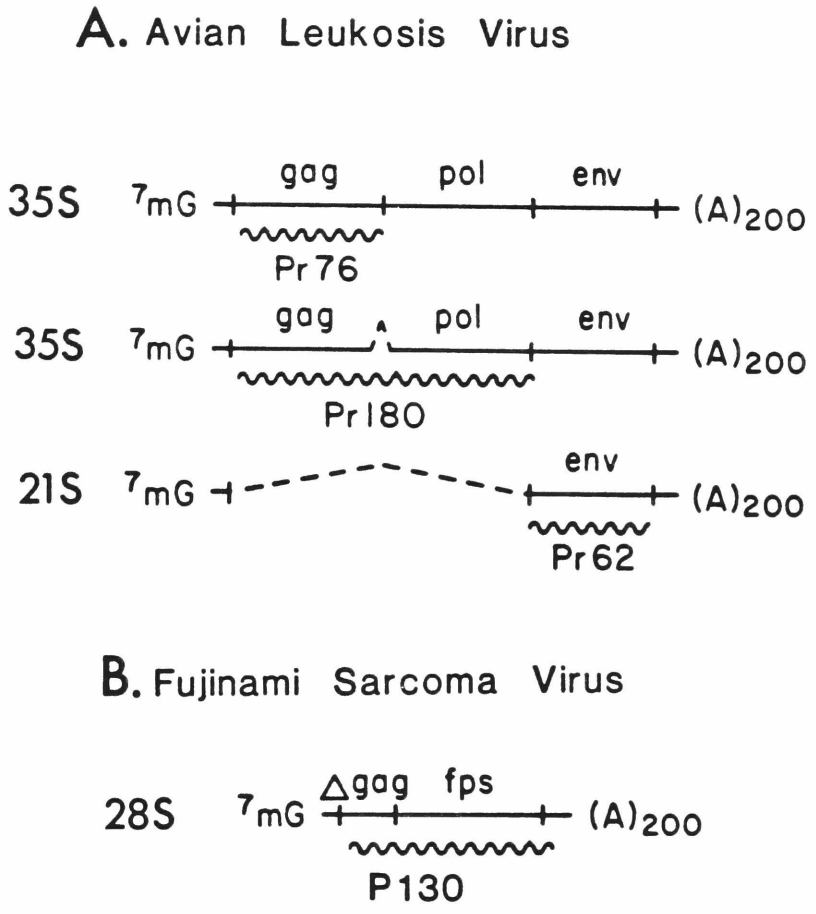


Figure 1. Schematic representation of ALV and FSV RNAs.
(A) Genomic (35S) and subgenomic (21S) RNAs of ALV. The 35S species contains the three structural genes gag, pol, and env. It directs the synthesis of the two precursor proteins $Pr76^{gag}$ and $Pr180^{gag-pol}$, indicated by the wavy lines. The spliced, subgenomic RNA contains the env gene and directs the synthesis of the precursor protein $Pr62^{env}$.
(B) FSV genomic RNA. The 28S RNA contains part of the gag gene fused to the fps sequence; it is translated into a polyprotein, P130 (wavy line), the transforming protein of FSV.
^{7}mG: cap structure; $(A)_{200}$: poly(A) tail.

The association of P140 with a protein kinase activity was first established in an in vitro assay, where an immune precipitate containing P140 was incubated in the presence of γ-^{32}P-ATP. The kinase activity of P140 resulted in the phosphorylation of tyrosyl residues of P140 itself, and of suitable substrates, such as α-casein [35]. P140 also acts as a protein kinase in vivo, since FSV-transformed cells are characterized by a 5-6 fold increase in the amount of phosphotyrosine in total cellular proteins [94, and this thesis].

Two lines of evidence point to the role of P140 in transformation by FSV. First, the isolation of mutants of FSV, which were temperature sensitive for transformation, showed that the lack of transformation at the non-permissive temperature correlated with a much reduced kinase activity of P140 (or P130) at that temperature [94, this thesis]. Second, the nucleotide sequence of a molecular clone of FSV revealed that P130 was the only product that could be translated from the large uninterrupted reading frame of genomic FSV RNA [117], and this was confirmed for a strain coding for P140, by in vitro translation [69].

In previous studies, a significant homology between fps, and fes the transforming gene of the Gardner-Arnstein and Snyder-Theilen strains of feline sarcoma virus (GA- and ST-FeSV), was revealed by nucleic acid hybridization and by immunological and tryptic peptide analyses of the respective onc gene products [5,6,114]. However, when the deduced amino acid sequences of these transforming proteins became available, the

full extent of homology was appreciated; about 70% of the amino acids between the fps and fes polypeptides were homologous when their unique regions were compared [47,117]. These results suggest that c-fes and c-fps, the cellular progenitors of the transforming genes in FeSV and FSV respectively, are cognate genes in different animal species.

Sequences of several transforming viruses have been cloned, and the complete nucleotide sequence obtained. It was thus possible to compare the deduced amino acid sequences of the different transforming proteins. A striking result emerged from these comparisons. The amino acid sequences of the transforming proteins of Rous sarcoma virus, Moloney murine sarcoma virus, Y73, and FSV showed varying extent of homology (23 to 80%) in their C-terminal portion, even though the respective transforming genes were not homologous, as judged by cDNA-RNA liquid hybridization [62,117,125,131]. Recently, this homology has been extended to another retroviral transforming protein, the abl protein of Abelson murine leukemia virus (D. Baltimore, personal communication). Interestingly, aside from the mos protein, all these proteins are associated with tyrosine kinase activity. This suggests the existence of a common ancestor gene, which was associated with the same enzymatic activity, and which subsequently diverged [56].

Scope of this thesis: a puzzling question that has emerged from the field of retroviruses is, how essentially a same gene can have a radically different effect as a cellular gene (c-onc gene) or as a viral gene (v-onc gene). It is known that high

expression of these viral genes lead to transformation, and also that their cellular counterparts are only expressed at low copy number, if at all. But, is transformation simply a matter of regulation of expression, or are there some subtle differences between the c-onc and v-onc genes that determine their oncogenic potential?

This thesis is a attempt to answer some of these questions, in the case of the avian virus, Fujinami sarcoma virus. First, we established that the enzymatic activity of the FSV transforming protein was essential for transformation. We then looked at the relationship between the c-fps and v-fps gene products, and at the possible function that the former might have in hematopoiesis in chickens. Finally, we analyzed the different modes of regulation of expression of v-fps in transformed cells, and this led us to propose a model of oncogenesis by FSV.

MATERIAL AND METHODS

Cells and viruses: Chicken embryo fibroblasts (CEF) were cultured as described [48], except that Ham's F-10 medium with 5% calf serum was used for all secondary cultures.
The isolation of Fujinami sarcoma virus (FSV), Fujinami associated virus (FAV), FSV NY225, FSV NY240 are the subject of previous reports [51,52]. Fisher rat embryo cells, 3Y1, transformed by FSV [77] were maintained in Dulbecco's modified Eagle's medium (Gibco), containing 10% fetal calf serum. Cells infected with AEV ts 34 [45] were a gift from S.M. Anderson. AMV-infected cells, clone BM-2 [82] were provided by J.H. Chen. Bone marrow cells were obtained from the femurs of 6-18 day old chickens according to the procedure of Fuller et al. [39].

Cloning of rat fibroblasts: Cultures to be cloned were trypsinized, single cells were picked under microscopic surveillance and placed into microtiter wells containing 0.1 ml of growth medium (10% fetal calf serum; Dulbecco's modified Eagle's medium). After three weeks, colonies were screened for morphology and transferred to 35 mm tissue-culture dish (LUX). Selected cultures were grown to mass culture and kept in the medium described above.

Drug treatment of rat fibroblasts: Fluorodeoxyuridine (FUdR), colcemid and hydroxyurea treatments were essentially as described [88,133].
FUdR: $2x10^6$ FSV-transformed 3Y1 cells were plated in a 250 ml

Falcon bottle in growth medium (see above). After a 24 h incubation, the medium was removed and the cells were treated for 1 h with 20 ml of fresh medium containing 10 ug/ml of FUdR. The cells were then washed twice with Tris-Glu buffer (25 mM Tris-HCl, pH 7.4; 135 mM NaCl; 5mM KCl; 0.4 mM Na_2PO_4; 5.5 mM glucose), trypsinized and plated on a 100 mm tissue culture dish. After 24 h, surviving cells were trypsinized and cloned as indicated.

Colcemid: $2x10^6$ FSV-transformed 3Y1 cells were seeded as above. 24 h later, the medium was removed and replaced with 20 ml of fresh medium containing 0.04 ug/ml of colcemid. Cells were incubated for 24 h at 37 C, and then handled as described after FUdR treatment.

Hydroxyurea: Confluent cultures of FSV-transformed 3Y1 cells were exposed in 100 mm plates for 48 h in growth medium containing 1 mM hydroxyurea. After drug treatment, surviving cells were trypsinized and split 1 to 2 in 100 mm plates. Cells were subcultures till they reached confluence, and then treated again with hydroxyurea under the same conditions. Cultures went through a total of three rounds of hydroxyurea selection. Surviving cells were trypsinized and replated at about 500 cells per 100 mm plates. After 10-18 days, colonies were screened for morphology and cells with altered phenotypes (flat, or semi-transformed) were selected for cloning.

5-azacytidine: 3Y1 cells or revertants of 3Y1 cells originally transformed with FSV, were plated at a density of $5x10^5$ cells per 100 mm tissue culture dish, in growth medium. 24 h after

seeding, cells were exposed to 12 ml of fresh medium containing either 5 uM or 15 uM 5-azacytidine for 48 h. After this treatment, cells were washed twice with growth medium and subcultured until they reached confluence. Cultures were then medium-changed every 3 days and examined for the appearance of foci.

Nomenclature of isolated clones: (See also Fig. 1, p. 77, for examples). The first numeral refers to the original focus from which a clone was derived.

Parental clone: for each single clone obtained after the first round of cloning, this numeral is followed by a capital letter (different from F or T). Each original clone was then treated with various drugs (as described above), and surviving cells were subcloned.

Subclones: the capital letter of the parental clone has been replaced by a numeral followed by the letter F (for flat morphology) in the revertants. When the revertants retransformed spontaneously, the letter T (for transformation) was used instead of F, in the cloned retransformants.

Superinfected clones: in clones superinfected with FSV, ST followed by a numeral was used instead of F, to distinguish these clones from spontaneous retransformants.

Bone marrow cells separation: Bone marrow cells were separated into several fractions on the basis of their density by centrifugation on density gradients of bovine serum albumin (BSA) according to Gazzolo et al. [41]. 4×10^6 bone marrow cells were

resuspended in 5 ml of growth medium and loaded on top of 5 ml of Ficoll-Paque (Pharmacia) in a 15 ml Corning tube. The cells were centrifuged at 1300 rpm (400 x g) for 30 min. Cells at the interface and half of the "fuzzy" layer just beneath the interface were pooled together, resuspended with two volumes of growth medium and centrifuged at 2000 rpm for 10 min. The cell pellet was resuspended in phosphate-buffered saline buffer (PBS: 0.795 g/L Na_2HPO_4; 0.144 g/L KH_2PO_4; 9 g/L NaCl) to a final density of $5x10^7$ cells per ml. 2 ml of this suspension were loaded on top of successive 1.5 ml layers of BSA solutions in concentrations decreasing by 2% from 33% to 19%, in a 15 ml Corning tube. Centrifugation was then carried at 700 x g for 30 min at 4 C. Cells present at the interface of the BSA layers were collected, washed once and resuspended in growth medium.

Isotopic labeling of cells and preparation of cell extracts: ^{35}S-methionine labeling was the following: FSV-infected CEF or rat 3Y1 fibroblasts, grown in 35 mm tissue culture plates were starved for 1 h in methionine free medium. The cultures were then changed to fresh medium (0.5 ml) containing 500 uCi/ml of ^{35}S-methionine (New England Nuclear, >500 Ci/mmol) and labeled for 4 h, or otherwise as indicated in the text. ^{32}P-orthophosphate labeling of cultures was as follows: cultures, grown in 60 mm plates were starved for 5 h in phosphate-free medium 199 (Gibco). The cultures were then changed to fresh medium (1.5 ml) containing 3 mCi/ml of ^{32}P-orthophosphate (New England Nuclear; carrier free) and incubated for 4 h or 13 h as

indicated. Bone marrow cells were labeled essentially in the same conditions, with minor modifications. ^{35}S-methionine labeling was done for 9 h in 3 ml of medium containing 1 mC/ml of the isotope; ^{32}P-orthophosphate labeling was for 2 h in 3.5 ml of medium 199 containing from 1 to 5 mCi/ml of the isotope (no significant differences were observed when either concentration was used).

Labeled cultures were washed three times with ice cold Tris-Glu buffer and lysed in modified NP-40 buffer (10 mM sodium phosphate, [pH 7.4]; 150 mM NaCl; 25 mM EDTA; 10% glycerol; 2 mM phenylmethylsulfonyl fluoride [PMSF]; 2% Trasylol; 0.5% Nonidet P-40 [NP-40]; 0.1% sodium deoxycholate) containing 0.1% 2-mercaptoethanol. 0.4 ml of lysis buffer was used for cultures kept in 35 mm plates, and 1.0 ml for cultures grown in 60 mm plates. Tissues were homogenized in the same buffer (4 parts buffer:1 part wet weight tissue). Cell lysates or tissue homogenates were transferred to a 1.5 ml Eppendorf microtest tube (Brinkman); samples were pipetted up and down five times through a blue Pipetman tip and centrifuged at 4 C for 6 min at 10,000 rpm in a Sorvall SE-12 rotor. The resulting clarified supernatants were used in all subsequent manipulations.

Immunoprecipitation: Serum from tumor bearing rabbits (TBR serum) induced by Schmidt-Ruppin D strain of Rous sarcoma virus, and rabbit antiserum raised against RAV-2 virion proteins (antivirion antiserum) were prepared as described [10,35,58]. Tumor regressor rat antiserum raised against FSV-transformed 3Y1 cells (anti-fps antiserum) was obtained as

described under Results. Preabsorbed antiserum was prepared by adding 50 ug of protein from disrupted purified RAV-2 in modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 25 mM EDTA; 1% Trasylol) to 5 ul of anti-fps antiserum, incubating at room temperature for 60 min, and spinning out the precipitate in a Beckman microfuge. Aliquots of cell extracts were incubated with 5-10 ul of antivirion or TBR antisera, or with 15-25 ul of anti-fps antiserum. Extracts of labeled or unlabeled cells were incubated with serum for 1 h on ice (when antivirion or TBR sera were used) and for 4 h when anti-fps was used. 25 ul of a Staphylococcus aureus protein A-Sepharose (Pharmacia) slurry (50% vol/vol in 20% glycerol) were added and mixed for 30 min to 90 min at 4 C to adsorb the immune complexes. The sepharose pellet was washed five times in modified NP-40 buffer and three times in 20 mM HEPES, 10% glycerol, pH 7.4. The pellet was resuspended in 50 ul of gel sample buffer or assayed for protein kinase activity.

Protein kinase assay: To the packed pellet of immune complex-protein A-sepharose was added 20 ul of reaction mixture: 50 mM HEPES (pH 77.4); 10 mM $MnCl_2$; 10% glycerol; 0.3-0.6 uM g-^{32}P-ATP (>2000 Ci/mmol, Amersham Corp.). The sample was mixed gently by tapping the tube several times, and incubated for 15 min at 25 C. When indicated, α-casein, at a concentration of 1 mg/ml was added at the start of the reaction. The reaction was stopped by addition of 50 ul of gel sample buffer.

Polyacrylamide gel electrophoresis: Polyacrylamide slab gels (30:0.8 acrylamide/bisacrylamide) containing 0.1% SDS were prepared according to the procedure of Laemmli [65]. Immunoprecipitates and kinase assay samples were heated in sample buffer for 3 min in boiling water, and loaded together with the sepharose beads onto the gel. Gels were run at 12 to 20 mA, constant current, until the tracking dye reached the bottom of the gel. Gels were not fixed or stained. They were dried onto filter paper before exposure to film for autoradiography in the presence of an intensifying screen. To locate ^{35}S-methionine labeled proteins, unfixed gels were treated with En^{3}Hance (New England Nuclear) according to the manufacturer's recommendations, dried onto filter paper and exposed on film. When the proteins in the gel had to be further processed (trypsin digestion, etc.), En^{3}Hance treatment was replaced by soaking the gel in 1mM salicylate for 30 min, before drying onto filter paper and exposure to film [14].

V8 protease analysis: Protein samples from excised gel bands were digested with the protease Staphylococcus aureus V8, essentially as described [11]. Gel bands were washed five times in 10% methanol, lyophilized to dryness, and loaded into a gel slot of a 12% polyacrylamide gel. Each band was overlaid with 75 ul of V8 sample buffer (62.5 mM Tris-HCl,pH 6.8; 0.15% SDS; 20% glycerol; 20 mM DTT; 0.01% bromophenol blue) containing S. aureus V8 protease at a concentration of 1 ug/ml. The electrophoresis was performed at 7.0 mA (constant current) until the dye front was well into the separating gel. The

current was then increased to 20 mA. The gel was dried down and processed for autoradiography.

Tryptic analysis of radiolabeled proteins: A protein band located by autoradiography or fluorography was excised from the gel, the paper scraped off and the gel slice was washed four times 15 min in 10% methanol, in a 1.5 ml Eppendorf microfuge tube (Brinkman). The washed gel slice was lyophilized to dryness and treated with 200-300 ul of cold performic acid (30% hydrogen peroxide and 88% formic acid, 1:19, preincubated 1 h at room temperature), for 1 h on ice. The sample was then lyophilized for 1 h, rehydrated in 0.5 ml of water, frozen and relyophilized to remove the remaining performic acid. To the dried, oxidized gel slice was added 0.3 ml of trypsin-TPCK (Worthington) at 50 ug/ml, in 50 mM ammonium bicarbonate, for 6 h at 37 C. The gel band was then crushed with a glass pestle and an additional 0.8 ml of trypsin solution was added overnight. A final 0.4 ml of freshly prepared trypsin solution was added for 6 h. Total digestion time was 24 h, at 37 C. The digested sample was millipore-filtered (Millex-GS, 0.22 um; Millipore Corp.) and the filter was rinsed with 1 ml of water. the combined fractions were lyophilized three to four times. The sample was dissolved in 50 ul water for a final lyophilization to concentrate the residue. The samples were redissolved in 10 ul of the appropriate electrophoresis buffer, and applied to 20 x 20 cm cellulose coated plates (E. Merck). ^{32}P-labeled peptides were resolved in two dimensions according to Feldman et al. [36]. Electrophoresis was performed in pH

1.9 buffer (acetic acid- formic acid-water, 78:25:897, vol/vol), at 1000 V for 1 h at 0 C. After air drying, the plates were developed in the second dimension by ascending chromatography at room temperature in buffer containing butanol- pyridine-acetic acid-water in the ratios 15:10:3:12. ^{35}S-labeled peptides were resolved according to Beemon [6]. Electrophoresis in the first dimension was carried out at pH 4.7 (butanol-pyridine-acetic acid-water, 2:1:1:36) at 1000 V for 30 min at 0 C. Ascending chromatography was in a buffer containing the same reagents at a ratio of 97:75:15:60. After drying, the plates were treated with En^{3}Hance spray (New England Nuclear) to facilitate visualization of ^{35}S-methionine labeled peptides. From 3000 to 9000 cpm of peptides were spotted on each plate.

Phosphoamino acid analysis: ^{32}P-labeled tryptic peptides were resuspended in 20 ul of 6N HCl. Hydrolysis was carried out in sealed capillary tubes at 100 C for 2 h. The resulting hydrolysates were diluted in 0.5 ml water and millipore-filtered once (Millex-GS, 0.22 um; Millipore Corp.). They were then lyophilized several times to remove HCl, and finally dissolved in 8 ul of pH 1.9 buffer (see above), containing cold standards of phosphoserine, phosphothreonine and phosphotyrosine at a concentration of 1 mg/ml each. The samples were spotted onto cellulose coated plates (E. Merck) and run at pH 1.9 , at 1000 V for 1 h at 0 C. The plates were air dried, and electrophoresed in the second dimension in pH 3.5 buffer containing acetic acid, pyridine and water in the ratios 10:1:189, for 1 h

at 0 C, at 1000 V. The air-dried plates were sprayed with a ninhydrin solution (0.5 g of ninhydrin in 100 ml of acetone containing 70 ul of pyridine), to identify the respective position of the cold standards. Comigration of ^{32}P label (detected by autoradiography) with the ninhydrin-stained standards positively identified the labeled amino acids.

The composition of phosphoamino acids in total cellular proteins was determined according to Hunter and Sefton [57], except that hydrolysis was for 2.5 h in vacuo. ^{32}P-labeled cells were lysed in RIPA buffer, and insoluble nuclear and cytoskeleton elements were removed by centrifugation. Phosphoproteins were then extracted twice with an equal volume of phenol saturated with 0.1 M NaCl; 50 mM Tris-HCl, pH 7.5; 5 mM EDTA. The combined phenol phases including the interface in both cases were diluted 1:40 with water and proteins were precipitated by addition of trichloroacetic acid to a concentration of 15%. The precipitate was recovered by centrifugation and extracted twice with a large volume of chloroform/methanol 2:1 (vol/vol). The resulting proteins were dissolved in 6N HCl and hydrolyzed for 2.5 h in vacuo. Phosphoamino acids were separated as outlined above.

Preparation of DNA and RNA: Isolation of high molecular weight DNA was done as described [115]. Cells grown in 100 mm tissues culture plates were trypsinized, washed once with Tris-Glu buffer and resuspended in 1 ml per plate of TEN buffer (0.1 M Tris-HCl, pH 7.4; 10 mM EDTA; 0.1 M NaCl) containing 0.5% SDS and 0.5 mg/ml of pronase (preincubated 1 h at 37 C). After in-

cubation at 37 C for 7 to 10 h, the samples were extracted twice with phenol-chloroform (1:1) and once with chloroform. DNA was spooled up after addition of NaCl to 0.2 M and two volumes of ethanol, and was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). The samples were treated with 50 ug of RNase A per ml (pretreated at 95 C for 5 min) at 37 C for 1 h, extracted with phenol, and precipitated with ethanol. DNA was dissolved in TE buffer and the concentration was examined by absorbance at 260 nm.

Isolation of total cellular RNA was as followed: Cells were trypsinized, washed once with Tris-Glu buffer and centrifuged at 1000 x g for 10 min. The cell pellet was lysed by addition of 8 ml of guanidium-thiocyanate buffer (4 M guanidium-thiocyanate; 0.1 M sodium acetate, pH 5.0; 5 mM EDTA), and this solution was layered on top of 4 ml of CsCl solution (0.84 g/ml CsCl; 0.1 M sodium acetate, pH 5.0; 5 mM EDTA) and RNA was pelleted by centrifugation at 33,000 rpm for 15 h in a SW-40 type rotor. The RNA pellet was resuspended in 0.5 ml of TE buffer, and RNA was ethanol precipitated after the addition of sodium acetate to 0.2 M. The RNA concentration was calculated by measuring the absorbance at 260 nm (we chose 1 O.D.^{260} to represent 50 ug/ml of RNA).

Nucleic acid hybridization: Hybridization between $cDNA_{fps}$ and cellular RNA was carried out under conditions of moderate stringency (50 C in 30% [vol/vol] formamide; 0.45 M NaCl; 45 mM sodium citrate; 5 mM EDTA and 0.1% SDS), and the extent of hybridization was determined by S1 nuclease digestion [53,115].

RNA samples (at concentrations ranging from 12.5 mg.ml to 0.02 mg/ml) were sealed in 50 ul capillary tubes, preheated at 95 C for 5 min, and then incubated at 60 C for 100 h. After incubation, the samples were frozen and stored at -70 C, or were processed directly. Extent of hybridization was assayed by S1 nuclease digestion. Samples were diluted into 0.4 ml of 25 mM potassium acetate, pH 7.4 containing 5 mM $ZnSO_4$, 0.3 M NaCl, 60 ug/ml of double-stranded salmon sperm DNA, and 0.04% SDS. One half of the diluted samples was kept at 0 C for estimation of total counts and the other half was digested with 10^4 U of S1 nuclease (Boehringer Mannheim Corp.) at 37 C for 2 h. Acid insoluble material in each half was collected on a glass fiber filter and counted in a scintillation counter. The number of copies of v-fps mRNA in transformed or phenotypically normal cells was calculated as followed: number of copies per cell = ($C_rt_{1/2}$ for viral RNA / $C_rt_{1/2}$ for cellular RNA) x (weight of RNA per rat cell / weight of one molecule of viral RNA), in which $C_rt_{1/2}$ indicates the C_rt value at which half-maximal hybridization is obtained. The amount of rat cellular RNA was determined in this study to be 10^{-11} g per cell. Shibuya et al. [114] showed that the $C_rt_{1/2}$ value of $cDNA_{FAV}$ for purified 35S FAV RNA (4.5×10^{-18} g per molecule) was about 0.02 under these hybridization conditions.

DNase I treatment: Nuclei isolation and DNase I treatment were performed essentially as described [80]. Rat fibroblasts were trypsinized and washed once with cold phosphate buffered saline (PBS) containing 5 mM EDTA. Cells were centrifuged at 1000 x g

for 5 min. The cell pellet was resuspended and washed three times (1000 x g, 5 min) in buffer A (100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 3 mM $MgCl_2$; 0.1 mM PMSF), washed three times (3000 x g, 5 min) in buffer A containing 0.2% Triton X-100, and then three times (1000 x g, 5 min) in buffer A without detergent. The nuclei were finally washed and resuspended in DNase I digestion buffer (100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 2 mM $MgCl_2$; 1 mM $CaCl_2$; 0.1 mM PMSF). The digestion was carried out at 37 C for 20 min with final concentrations of DNase I ranging from 0 ug/ml to 0.8 ug/ml. The nuclei suspension in each digest was adjusted to an $O.D._{.}^{260}$= 10 (or a nuclear concentration of DNA of approximately 0.5 mg/ml). After DNase I digestion, the samples were adjusted to 0.5 mg/ml of pronase and 0.5% SDS. DNA was extracted as outlined earlier.

Southern blotting analysis: 10-15 ug of high molecular weight cellular DNA, obtained from rat fibroblasts, were digested with 20 to 30 U of several restriction endonucleases (New England Biolabs), concentrated by ethanol precipitation and separated by electrophoresis in 0.8% or 1.5% agarose gels in a buffer solution containing 40 mM Tris-acetate (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA. The DNA was denatured neutralized in situ, and transferred onto a nitrocellulose sheet (Millipore Corp., pore size 0.22 um) with the use of 20 x SSC (1 x SSC: 0.15 M NaCl, 15 mM sodium citrate) as described by Southern [121]. The sheet was baked (75 C, in vacuo), preincubated at 37 C for a minimum of 1 h in a prehybridization solution (50% formamide [vol/vol]; 5 x SSC; 20 mM Tris-HCl, pH 7.4; 0.5% SDS)

and hybridized for 60-72 h at 37 C in the same buffer containing 250 ug/ml of high molecular weight salmon sperm DNA and a ^{32}P-labeled probe [70]. Typically between 10^6 to 10^7 cpm were used for hybridization. Filters which were to be rehybridized to a second radioactive probe were soaked at 65 C for 30 min in 1/15 x melting buffer (1 x melting buffer: 5o mM Tris-HCl, pH 7.4; 2 mM EDTA; 0.5% SDS; 1 x Denhardt's solution [0.02% each of bovine serum albumin, Ficoll, and polyvinyl pyrolidone]); the filter was blotted dry and incubated for another 30 min at 65 C in 1/15 x melting buffer containing 50% formamide (vol/vol).

Radioactive probes: Nick translation was performed as described by Maniatis et al. [75]. The labeled nucleotide used was α-^{32}P-dCTP at 10 mCi/ml in aqueous solution (specific activity > 2000 Ci/mmol, Amersham Corp.), and DNA polymerase I was obtained from New England Nuclear; specific activities of 5 x 10^7 to 4 x 10^8 cpm/ug of DNA were obtained.

5' end-labeled probes were obtained by treating the purified DNA fragment with bacterial alkaline phosphatase followed by incubation with T4 polynucleotide kinase and γ-^{32}P-ATP (specific activity >3000 Ci/mmol, Amersham Corp.).

Preparation of ^{3}H-labeled $cDNA_{fps}$ was as described [114], and was a generous gift from M. Shibuya, in this laboratory.

(i). FSV-representative probe (FSV_{rep}): total FSV DNA, purified from clone λ-FSV-2 [116] after digestion with Sac I and separation on 0.8% agarose gel, was electroeluted and phenol extracted once, before nick translation.

(ii). gag probe: the Eco RI fragment of the PSRA clone [28] containing the long terminal repeats (LTR) and part of gag was subcloned in pBR322 by F. Cross in this laboratory (clone PSC3). Purified Eco RI fragment DNA from this subclone was a gift from L.-H. Wang also in this laboratory. It was digested with Bam Hl, and the purified Bam Hl-Eco RI fragment containing gag specific sequences was nick translated.

(iii). Bam Hl-Sac II 3' viral probe: FSV subclone pBR-F16 [116], containing LTR-gag, was digested with Sac II; the DNA was ethanol precipitated, resuspended in Bam Hl digestion buffer and cut with this enzyme. The fragments were separated on a 5% acrylamide gel (acrylamide/bisacrylamide; 49:1) and stained with ethidium bromide. The Bam Hl-Sac II band was cut, DNA was electroeluted, phenol extracted and used for nick translation.

(iv). Bam Hl-Sac I viral probe: pBR-F16 DNA was cut with Bam Hl, ethanol precipitated, resuspended in Sac I digestion buffer and digested with Sac I. The Sac I-Bam Hl fragment was isolated by the same procedure as above, phenol extracted once and used for nick translation.

(v). Sau 3A-Hpa II 5' viral probe: pBR-F16 DNA was digested with Bam HI, and the viral DNA fragment purified. The DNA was then digested with Hpa II, the products were ethanol precipitated, resuspended in the appropriate buffer and 5' end labeled as outlined earlier. The labeled fragments were separated on a 5% polyacrylamide gel of the same composition as above and visualized by autoradiography. The appropriate fragment (about

1 kb in length) was electroeluted and ethanol precipitated after addition of 2 ug of carrier tRNA. The pellet was resuspended in Sau 3A digestion buffer, and treated with Sau 3A. The labeled 112 bp Sau 3A-Hpa II fragment was electroeluted and used for hybridization after heat denaturation (10 min at 95 C).

- I -

CHARACTERIZATION OF TEMPERATURE SENSITIVE MUTANTS OF FUJINAMI SARCOMA VIRUS.

Mutants of RNA tumor viruses which are temperature sensitive (ts) in cell transformation have been extremely useful in analyzing the process of transformation by these viruses. First, the existence of mutants which are sensitive only for transformation provided the first clear indication that the product of a viral gene is directly responsible for cellular transformation [38,49,76,134,145]. Second, ts mutants are useful in elucidating the biochemical events involved in transformation, particularly when the product of a transforming gene is associated with an enzyme activity, such as kinase activity [20,33,71, 107]. Thus, ts mutants of Rous sarcoma virus (RSV) were used to demonstrate that the expression of the kinase activity of $p60^{src}$, the transforming protein of RSV, was necessary for transformation [57]. In addition, a few in vivo substrates of $p60^{src}$ were identified, by the fact that they associated very tightly with the mutant $p60^{src}$, or that phosphorylation of these proteins at tyrosine residues was a function of the temperature of incubation at which the ts mutant-infected cells were maintained [11,99].

When this study was started, Fujinami sarcoma virus (FSV) had just been characterized. In non-producer cells transformed by FSV, P140 was the only viral protein that was detected [35,51,69]. Furthermore, it was associated with a kinase ac-

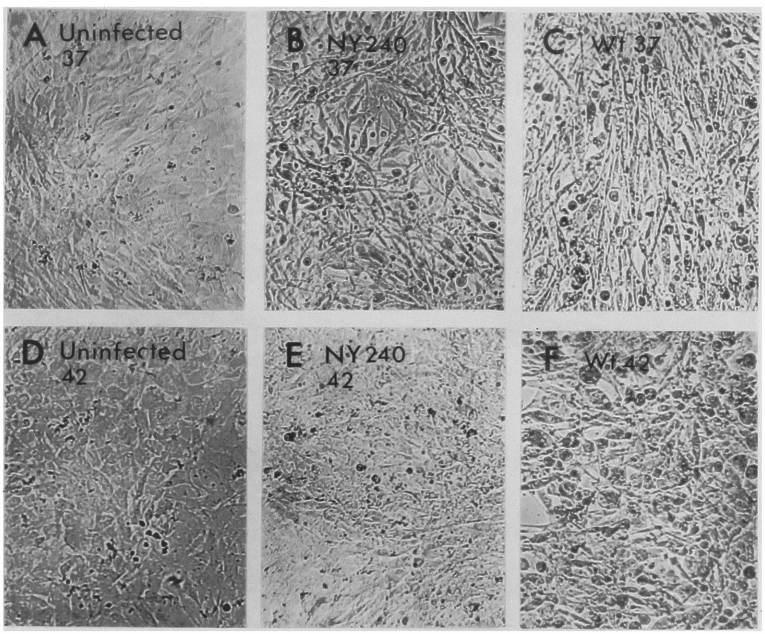
tivity greatly similar to that of $p60^{src}$, making P140 a likely candidate for the transforming protein of FSV [35]. Shortly after the initial characterization of P140, T. Hanafusa et al. [52] isolated two ts mutants of FSV. Extending the biological determination of these mutants, we show here, that P130 is the transforming protein of FSV. In ts-mutant infected cells, P130 was synthesized regardless of the temperature of incubation, but only at the permissive temperature could a functional kinase activity be observed. The inactivation of the protein kinase activity of P130 at the non permissive temperature correlated with the lack of transformation of ts mutant-infected cells kept at that temperature.

RESULTS

Two ts mutants of Fujinami sarcoma virus (FSV), FSV NY225 and FSV NY240, were isolated by T. Hanafusa after mutagenesis of an original FSV stock [52]. Infection of CEF with these mutants resulted in a transformed morphology at 37 C, the permissive temperature, identical to that of cultures transformed with wild-type FSV. At 42 C, the nonpermissive temperature, the mutant-infected cells displayed a morphology typical of uninfected CEF (Fig. 1). For both mutants, changes in morphology were reversible and could be obtained by shifting the temperature up and down several times. The disappearance of the morphological transformation by shifting to higher temperature often required more than 24 h, whereas transformation by shifting to a lower temperature was usually complete within 24 h. The ability of these mutants to produce foci and colonies at permissive and nonpermissive temperature is profoundly suppressed at 42 C. When mutant-infected cells were held at 42 C, they produced normal amount of FSV, which made foci only at the permissive temperature. Finally two biological parameters of FSV-transformed cells, sugar uptake and plasminogen activator production, were also found to be temperature sensitive in these mutants [52].

In order to determine the nature of the lesion in these mutants, we undertook the biochemical characterization of the mutant protein synthesized by FSV NY225 and NY240.

Figure 1. Photomicrographs of uninfected, FSV wild-type-infected, and ts mutant-infected cells. Chicken embryo cells were infected with FSV wild-type or NY240. Once fully transformed, these cultures and uninfected cultures were sub-cultured and incubated at 37 C or 42 C for 2 days. Uninfected cells at 37 C (A) and 42 C (D); NY240-infected cells at 37 C (B) and 42 C (E); and wild-type-infected cells at 37 C (C) and 42 C (F).


A Uninfected
37
B NY240
37
C WT 37
D Uninfected
42
E NY240
42
F WT 42

Transforming protein of the ts mutants: In the initial characterization of FSV, T. Hanafusa et al. reported that this virus coded for a transforming protein of 140,000 daltons, P140 [51]. When we characterized the protein encoded by FSV wild-type, which was used for the isolation of the two mutants, we detected a slightly smaller protein, P130, with a molecular weight of 130,000 daltons (Fig. 2). The change in the molecular weight of the virus protein is due to the existence of two FSV strains. The wild-type virus had been selected, after cloning of the original stock, on the basis that it produced more distinct foci at 41 C than other clones. We presume that the difference seen in this strain is due to a deletion, probably at the gag-fps junction of the viral genome (M. Shibuya, personal communication). Although the ts mutant-infected cells, maintained at 42 C, produced the same amount of virus as parallel cultures kept at 37 C, we wanted to determine the amount of P130 produced in these cells either at 37 or 42 C. Cultures infected with the ts-mutants were labeled for 9 h with ^{35}S-methionine at 37 or 42 C, along with cultures infected with the parental wild-type FSV. As shown in Fig. 3, the amount of P130 was identical at the two temperatures, both for ts or wild-type infected cells.

Figure 2. Comparison of the size of FSV proteins. Cells transformed with FSV (FAV) stock (a) and a clonal FSV (FAV) used as wild-type in this study (b) were labeled with ^{35}S-methionine for 5 h. Cells were lysed with RIPA buffer, and FSV proteins were immunoprecipitated with antiserum against RAV-2 proteins. The immunoprecipitates were analyzed by electrophoresis in a 5 to 15% gradient SDS-polyacrylamide gel, followed by fluorography. In addition to FSV P140 (a) or P130 (b), gag-containing products of helper virus (Pr180, Pr76, p27, p19, p15 and p12) were precipitated by the anti-RAV-2 serum.

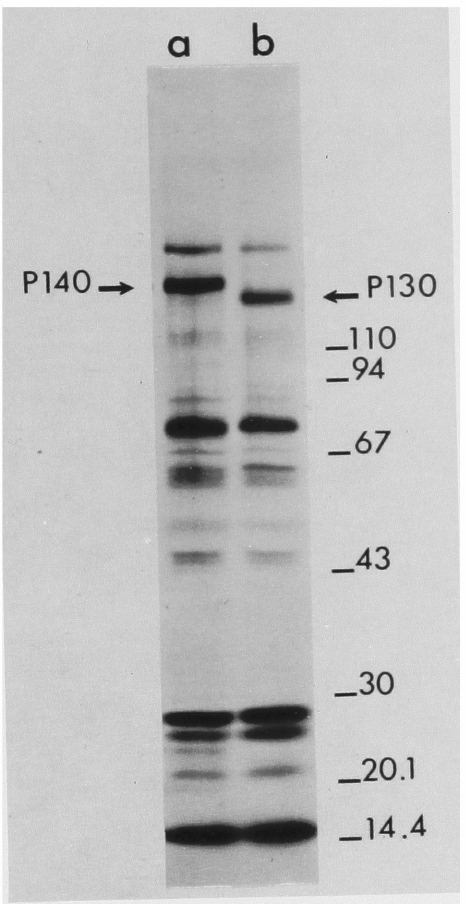
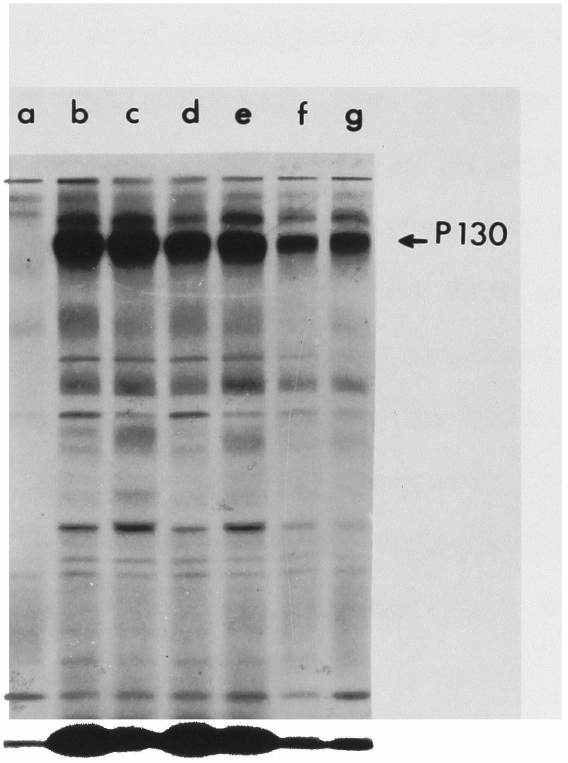
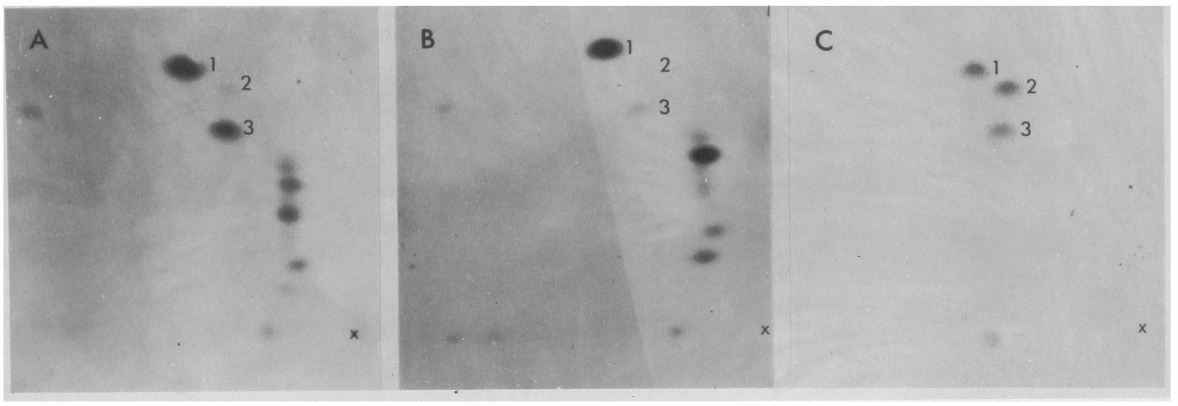

a
b
P140
P130
110
94
67
43
30
20.1
14.4

Figure 3. In vivo labeling of wild-type and ts mutant P130 with ^{35}S-methionine. Wild-type- and ts mutant-infected chicken cells were labeled with ^{35}S-methionine for 9 h. The labeled P130's were extracted with RIPA buffer, immunoprecipitated and analyzed by 8.5% SDS-polyacrylamide gel electrophoresis followed by fluorography. Uninfected cells at 37 C (a); cells infected with NY240 at 41 C (b), with NY240 at 37 C (c), with NY225 at 41 C (d), with NY225 at 37 C (e), with wild-type at 41 C (f), and with wild-type at 37 C (g).


a b c d e f g
P130

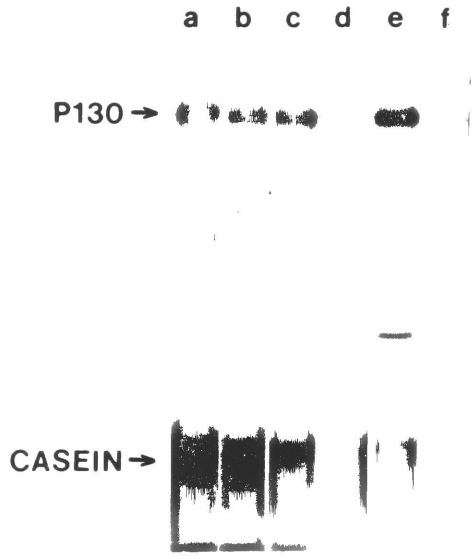
Phosphorylation of ts mutant P130 in vivo: To examine the state of in vivo phosphorylation of the mutant encoded P130, wild-type and mutant-infected cells were labeled with ^{32}P-orthophosphate at 37 and 42 C for 13 h. ^{32}P-labeled proteins were immunoprecipitated with antivirion antiserum and separated on a polyacrylamide gel. The P130 band was cut out from the gel, and the phosphoamino acid composition of P130 was analyzed after acid hydrolysis of the protein. Fig. 4 shows only the results obtained with P130 isolated from FSV NY240-infected cells, yet the same results were obtained when FSV NY225 was used. The acid hydrolysate of P130 isolated from cells kept at 37 C contained both phosphoserine and phosphotyrosine (Fig. 4A), whereas the amount of phosphotyrosine in P130 isolated from parallel cultures kept at 42 C, was severely reduced (Fig. 4B). No difference in phosphoamino acid composition of P130 in cells kept at either 37 or 42 C and infected with wild-type FSV, was detected (data not shown).

Figure 4. Analysis of the phosphoamino acid composition of NY240 P130 phosphorylated in vivo. Partial acid hydrolysates of the NY240 P130 labeled in vivo with $^{32}P_i$ were separated in two dimensions: electrophoresis at pH 1.9 was carried out from right to left, and electrophoresis at pH 3.5 was done from bottom to top. The origin is marked X. Standard phosphoamino acids : 1, phosphoserine; 2, phosphothreonine; 3, phosphotyrosine were electrophoresed on a separate plate (C) and their position was identified by staining with ninhydrin. (A) NY240 P130 from cultures kept at 37 C; (B) NY240 P130 from cultures kept at 42 C. The ^{32}P-labeled spots in the lower right of panels (A) and (B) are partially hydrolyzed phosphopeptides.


A
1
2
3
x
B
1
2
3
x
C
1
2
3
x

Since the FSV transforming protein was known to autophosphorylate in vitro and presumably in vivo also, this decrease in phosphotyrosine level at 42 C in ts-mutant P130 might be the result of the inactivation of its enzymatic activity at that temperature. Therefore, cell extracts of ts mutant-infected cells kept at 37 and 42 C were immunoprecipitated with antivirion antiserum and the immune complexes were assayed for kinase activity in the presence of the in vitro substrate, α-casein (Fig. 5). The protein kinase activity was significantly reduced when extracts of ts mutant-infected cells, maintained at 42 C, were assayed (5A, B; lanes d,f), whereas no difference in activity was noticed at either temperature with wild-type-infected cells (5A, lanes a,b). The reduced phosphorylation of α-casein as well as P130 suggests a decrease in the enzyme activity rather than the reduction of the phosphate-accepting capacity of P130 as a result of a configurational alteration. A comparison was also made for the enzyme activity extracted with RIPA or modified NP-40 buffers. The kinase activity was slightly better preserved in the latter buffer, but the ratio of the activity at the two temperatures remained basically the same.

Figure 5. Protein kinase activity of P130 assayed in extracts of cultures infected with wild-type FSV, NY225, or NY240. Immunoprecipitates from infected cells kept at 37 C (lanes a, c and e) or 42 C (lanes b, d and f) were tested for protein kinase activity in vitro. The reaction mixture contained o.5 mg of α-casein per ml as a substrate. $^{32}P_i$ was tranferred from γ-ATP to both P130 and α-casein molecules. Cell lysates were prepared in RIPA buffer. Lanes a and b, wild-type infected cells; lanes c and d, NY225-infected cells; lanes e and f, NY240-infected cells.


a b c d e f
P130 →
CASEIN →

Phosphorylation of tyrosine residues of cellular proteins in vivo: Hunter and others showed that phosphorylation of tyrosine residues is elevated in Rous sarcoma virus-transformed cells, and that this increase (6 to 7 fold) correlated with transformation by retroviruses coding for tyrosyl kinases as transforming proteins [6,57,111]. To confirm that the protein kinase activity in FSV ts mutant-infected cells is temperature dependent also in vivo, we measured the level of phosphotyrosine in NY225 infected cells (Table 1). As expected, the levels at permissive and nonpermissive temperatures were equivalent to those of wild-type-infected and uninfected cells, respectively.

Table 1. Level of phosphoamino acids in cells[a]

Cells	Temp (°C)	% of total cpm		
		Phosphoserine	Phosphothreonine	Phosphotyrosine
Uninfected	37	92.6	7.3	0.06
FSV wild type	42	92.1	7.6	0.30
FSV NY225	37	91.4	8.3	0.26
FSV NY240	42	93.7	6.2	0.08

[a]Cells were labeled with ^{32}P, for 18 h. The proteins were extracted and hydrolyzed with 6N HCl. Acid hydrolysates containing about 2×10^5 cpm were mixed with 10 µg each of phosphoserine, phosphothreonine, and phosphotyrosine. The mixture was subjected to two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension. The thin-layer plates were autoradiographed, and the radioactive spots that comigrated with phosphoamino acid markers, which were identified by ninhydrin, were scraped, and the radioactivity was measured.

DISCUSSION

The two ts mutants of FSV described here are very similar to T class ts mutants of RSV in a number of ways [49,134]. Cells infected with the FSV mutants were converted to either a transformed or a nontransformed state simply by shifting the incubation temperature. The reversible changes were observed with all the parameters of cell transformation that we examined. As a minor difference from RSV mutants, the time period required for morphological alteration by shifting temperature of FSV mutant-infected cells seems to be longer than that required for cells infected with representative RSV mutants [2,7,59,76,130,146]. The kinetics of morphological conversion should be studied further, because denaturation or reactivation of the protein kinase associated with P130 is complete within 1 hour and does not require protein synthesis (unpublished results). At any rate, the temperature sensitivity of the two FSV mutants was profound; there was no sign of a significant rate of back reversion. The virus production in mutant-infected cells at nonpermissive temperature was not appreciably lower than that at permissive temperature. This property cannot be directly compared, however with the case of T class RSV mutants [7,59,76,146], because the virus replicative functions of FSV are defective, whereas those of RSV are nondefective. The production of infectious FSV is the result of the formation of pseudotypes, which have the FSV genome encapsidated in virion particles, with all other virion proteins encoded by a helper virus.

Cells infected with FSV mutants produced P130 at both permissive and nonpermissive temperatures. The analysis of phosphoamino acids of in vivo ^{32}P-labeled ts mutant P130 showed that at the nonpermissive temperature P130 was not phosphorylated at tyrosine residues. The lack of tyrosine phosphorylation in vivo can be explained by the loss of enzyme activity of the mutant P130 as determined by the in vitro assay. As we have shown, the protein appeared to be active as a protein kinase, only when extracted from cultures incubated at the permissive temperature. In addition, conformational changes in ts mutant P130 at the nonpermissive temperature, may also contribute to the lack of phosphorylation of its own tyrosine sites. We attempted to see whether NY240 P130 isolated from cells kept at 42 C could be phosphorylated in trans by wild-type P140. FSV proteins were immunoprecipitated from a mixture of cell extracts, made from NY240-infected cells kept at 42 C and from wild-type-transformed cells, and the extent of phosphorylation of NY240 P130 was monitored after the in vitro kinase assay. There was no significant increase in the phosphorylation of P130, and this suggests that the mutant P130, made at 42 C, fails to serve as an in vitro substrate for P140. However, this conclusion is valid if the mechanism of autophosphorylation is trans, and if steric factors do not prevent P140 from acting on P130.

Sefton et al. [110] showed that p60^{src} of cells transformed by some RSV ts mutants is enzymatically more active when it is extracted with NP40 buffer rather than RIPA buffer.

Comparison of cell extracts made in the two buffers which were different in their detergent composition showed that the enzymatic activity of P130 of FSV mutants was not particularly labile in the presence of the ionic detergent, SDS. Therefore, we consider the overall results to indicate that the level of enzyme activity of P130 extracted with the two buffers from ts mutant-infected cells correlates with the level of P130 protein kinase activity within the cells. This conclusion was further supported by the fact that the level of phosphotyrosine in total cell protein was increased, only when NY225-infected cells were incubated at the permissive temperature (Table 1). Moreover, the absence of elevated level of phosphotyrosine in mutant-infected cells maintained at the nonpermissive temperature is unlikely to result from an intracellular redistribution of P130, away from its cellular substrates. Recently, Feldman et al. have reported that the intracellular location of P130 in rat cells non-productively infected with FSV, is predominantly cytoplasmic, with P130 also present at some areas of the plasma membrane [37]. This cytoplasmic distribution was not changed in cells infected with FSV NY225 or NY240 at either permissive or nonpermissive temperature. Thus, the analysis of the FSV ts mutants seems to indicate that P130 (or P140) is the transforming protein, and that its enzyme activity plays an essential role in FSV-induced cell transformation.

- II -

NCP98: A CELLULAR PROTEIN HOMOLOGOUS TO THE v-fps GENE PRODUCT

Retroviral transforming genes (v-onc genes) are homologous to cellular genes (c-onc genes). Although, much information has been gathered about viral transforming proteins (structure, function, localization and expression), very little is known about the cellular products homologous to these transforming proteins. Liquid hybridization studies between cDNA specific for v-onc genes and cellular RNA indicate that c-onc mRNA is present at very low level in tissue of uninfected animals [83,115]. When possible, this was confirmed at the protein level, by using antisera with broad reactivity against the unique sequence of the viral transforming proteins; this allowed the identification of four such proteins: $p60^{c\text{-}src}$ homologous to Rous sarcoma virus $p60^{v\text{-}src}$ [19,89], NCP150 homologous to Abelson leukemia virus P120 [142], NCP92 homologous to ST-FeSV P85 [3] and $p21^{c\text{-}ras}$ homologous to Harvey sarcoma virus $p21^{v\text{-}ras}$ [66,112]. These immunologically cross-reactive cellular proteins were found to be structurally related to their viral counterparts. In addition, these proteins were shown in two cases to be functionally related to their viral homologs: $p60^{c\text{-}src}$ is associated with a protein kinase activity similar to that of $p60^{v\text{-}src}$ [21,58,89], whereas $p21^{c\text{-}ras}$ has the same nucleotide binding property as $p21^{v\text{-}ras}$ [107]. Whereas $p60^{c\text{-}src}$ and NCP92 seemed to be expressed constitutively at similar low levels in different tissues, NCP150 expression was elevated

in lymphoid tissue and one hemopoietic precursor cell line showed extremely elevated levels of $p21^{c\text{-}ras}$ suggesting a strong tissue specificity in the expression of some of those proteins [108,142].

Shibuya et al. [115] reported that c-fps mRNA was very low in most tissues of young chickens. The amount was, however, variable in different tissues, and highest in bone marrow, with two to three copies per cell. To identify the product of the c-fps gene, and study its distribution in various tissues, we have used a tumor-bearing rat antiserum specific for the unique sequence of FSV protein. It reacted specifically with a normal cellular protein of MW 98,000 (NCP98) that was immunologically, structurally and enzymatically related to FSV P140 and that showed a tissue specificity in its expression.

RESULTS

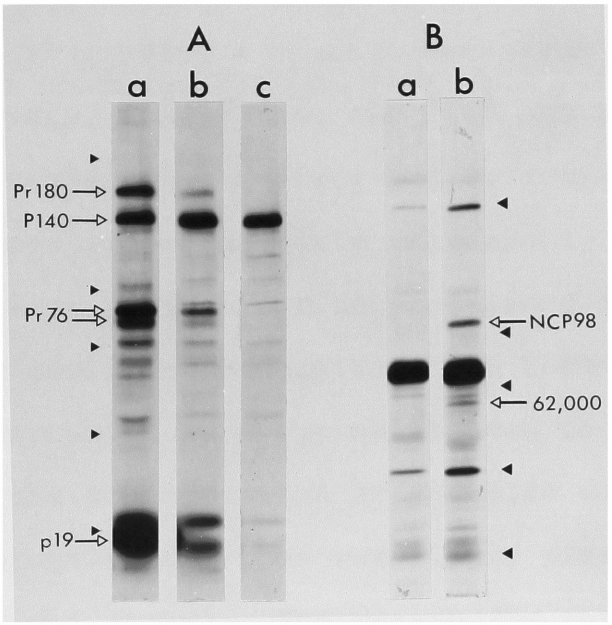
Tumor regressive rat antiserum specific against the transforming sequence of P140: In the initial characterization of FSV P140, Feldman, in our laboratory, had used an antiserum raised against structural virion proteins, that specifically reacts with the gag sequence present at the N-terminus of P140 [35]. This antiserum was unfortunately of no help to identify the product of the c-fps gene, which lacks any gag sequence. This meant that we had to develop an antiserum which would be directed against the fps sequence of P140, and hope that such an antiserum would crossreact with the product of the cellular gene, c-fps. At about that time, a line of rat fibroblasts (line 3Y1) was successfully infected with FSV in our laboratory [77]. These nonproductively-infected cells expressed no other viral protein than P130, the transforming protein encoded by the FSV strain which had been used for infection of the 3Y1 cells .

When 10^5-10^6 FSV-transformed 3Y1 cells were injected subcutaneously into adult Fischer rats, a tumor could be palpated after two to three weeks, at the site of injection. The tumor grew steadily for about 5 weeks, and then started to regress. Rats were bled at 1 week after the appearance of the tumor and then every 11-13 days. The specificity of the rat antiserum was tested after each bleed, for its ability to immunoprecipitate ^{35}S-methionine labeled P140. Aliquots of ^{35}S-methionine labeled extract from FSV-infected chicken embryo fibroblasts

were immunoprecipitated with antivirion antiserum or rat antiserum. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Both antisera were able to immunoprecipitate P140 as well as the gag precursor protein Pr76, the gag-pol precursor Pr180 and mature gag proteins p27 and p19 (Fig. 1A, lanes a, b). In order to remove antibodies against the gag-related proteins, we absorbed the rat antiserum with RAV-2 virion proteins. As shown in Fig. 1A, lane c, the absorption of the rat antiserum affected only slightly the precipitation of P140, whereas it abolished the precipitation of Pr76, Pr180, p27 and p19. Absorption of the rat antiserum with increasing amount of RAV-2 proteins did not decrease further the amount of P140 brought down by the antiserum (data not shown). From these results we conclude that the unabsorbed tumor-bearing rat antiserum, which we will call anti-fps, in addition to anti-gag determinants, contains antibodies that react specifically with the domain of P140 that is encoded in the unique sequence of FSV.

To establish further the specificity of this antiserum, we examined its cross-reactivity with the proteins of other avian sarcoma viruses (Rous sarcoma virus, Y73 and UR2). Since all three viruses are known to code for a unique transforming protein associated with protein kinase activity [20,36,60,71], we assayed the kinase activity in immunoprecipitates of cell extracts from cultures transformed with these viruses using anti-fps antiserum.

Figure 1. Selective precipitation by anti-fps of P140 from FSV-infected chicken embryo fibroblasts and of a normal cellular protein from bone marrow cell.
(A) Cell extract from FSV-transformed chicken embryo fibroblasts, labeled with ^{35}S-methionine, immunoprecipitated with the indicated antisera and analyzed on 5%-15% gradient SDS-polyacrylamide gels. Lane a, antivirion antiserum; lane b, FSV-specific tumor-bearing rat antiserum (anti-fps); lane c, anti-fps absorbed with Rous-associated virus 2 (RAV-2) proteins.
(B) Extract from uninfected chicken bone marrow cells labeled with ^{35}S-methionine and immunoprecipitated with non immune rat antiserum (lane a), or anti-fps (lane b). Arrows: NCP98 and the 62,000 dalton protein; viral structural proteins are also indicated (the FSV stock that was used contained two helpers that encode two slightly different Pr76 proteins). Arrowheads: molecular weight markers: myosin (H-chain; 200,000 daltons); phosphorylase b (92,500 daltons); bovine serum albumin (68,000 daltons); ovalbumin (43,000 daltons); d-chymotrypsinogen (25,700 daltons).


A
a
b
c
B
a
b
Pr 180
P140
Pr 76
p19
NCP98
62,000

In all cases we were unable to detect any phosphorylated protein, suggesting that anti-fps does not have determinants against the unique sequences of Y73 P90, Rous sarcoma virus $p60^{src}$ or UR2 P68. Since $p60^{src}$ might not be able to phosphorylate the IgG of anti-fps, we also examined whether anti-fps can immunoprecipitate $p60^{src}$ from ^{35}S-methionine-labeled extract of CEF transformed by Rous sarcoma virus. Again we could not detect $p60^{src}$ (data not shown).

Barbacid et al., using a caprine antiserum specific against FeSV protein, showed that FSV P140 and the transforming proteins of the Snyder-Theilen (ST) and Gardner-Arnstein (GA) strain of FeSV were immunologically related [5] . When we tested our antiserum against extracts obtained from GA-FeSV infected cells, we could not precipitate the FeSV protein of MW 115,000 (P115) expressed by these cells even though we were able to confirm the precipitation of FSV P140 using a rat anti GA-FeSV specific antiserum, that was kindly provided by J. R. Stephenson (data not shown). The reason for this discrepancy is not clear. Anti-fps is perhaps directed against a region that is not as strongly conserved in FeSV, or alternatively, the bulk of the antibodies present in anti-fps may react with sequences absent in P115, especially in view of the fact that the unique sequence of FSV, v-fps, is about 2.6 kb [116,117], whereas the transforming sequence of GA-FeSV v-fes, is about 1.8 Kb [34,47].

A protein from uninfected chicken bone marrow cells is immunologically cross-reactive with FSV P140: Recently, Shibuya et

al. [115] showed that the amount of c-fps mRNA is variable in different tissues of uninfected chickens, and highest in bone marrow cells (2-3 copies per cell). Therefore, bone marrow cells were a good candidate to study whether the product of c-fps mRNA could be detected by anti-fps. Uninfected bone marrow cells obtained from 8 day old chickens were labeled with ^{35}S-methionine and a cell extract was immunoprecipitated with either non-immune rat antiserum, or anti-fps. The immunoprecipitated proteins were then separated by gel electrophoresis.

As can be seen in Fig. 1B, anti-fps but not non-immune serum detected a protein of MW 98,000 as well as a minor band of MW 62,000. The heavily labeled band of MW 70,000 present in both lanes was not consistently seen in subsequent experiments. This band appears to be non-specific since it was also present when anti-gag antiserum or tumor bearing rabbit serum (TBR) were used to immunoprecipitate the bone marrow cell extract (data not shown). A band of MW 200,000 and comigrating with a myosin H chain marker appears to be nonspecifically precipitated by both immune and non-immune sera.

When a constant amount of ^{35}S-methionine-labeled bone marrow cell extract was mixed with unlabeled extract from FSV transformed CEF and immunoprecipitated with absorbed anti-fps, only the 98,000 dalton protein diminished in intensity with increasing amount of unlabeled P140 whereas the intensity of the protein of MW 62,000 did not vary significantly (data not shown). Since CEF did not expressed any detectable amount of the 98,000 dalton protein, this experiment confirms the immuno-

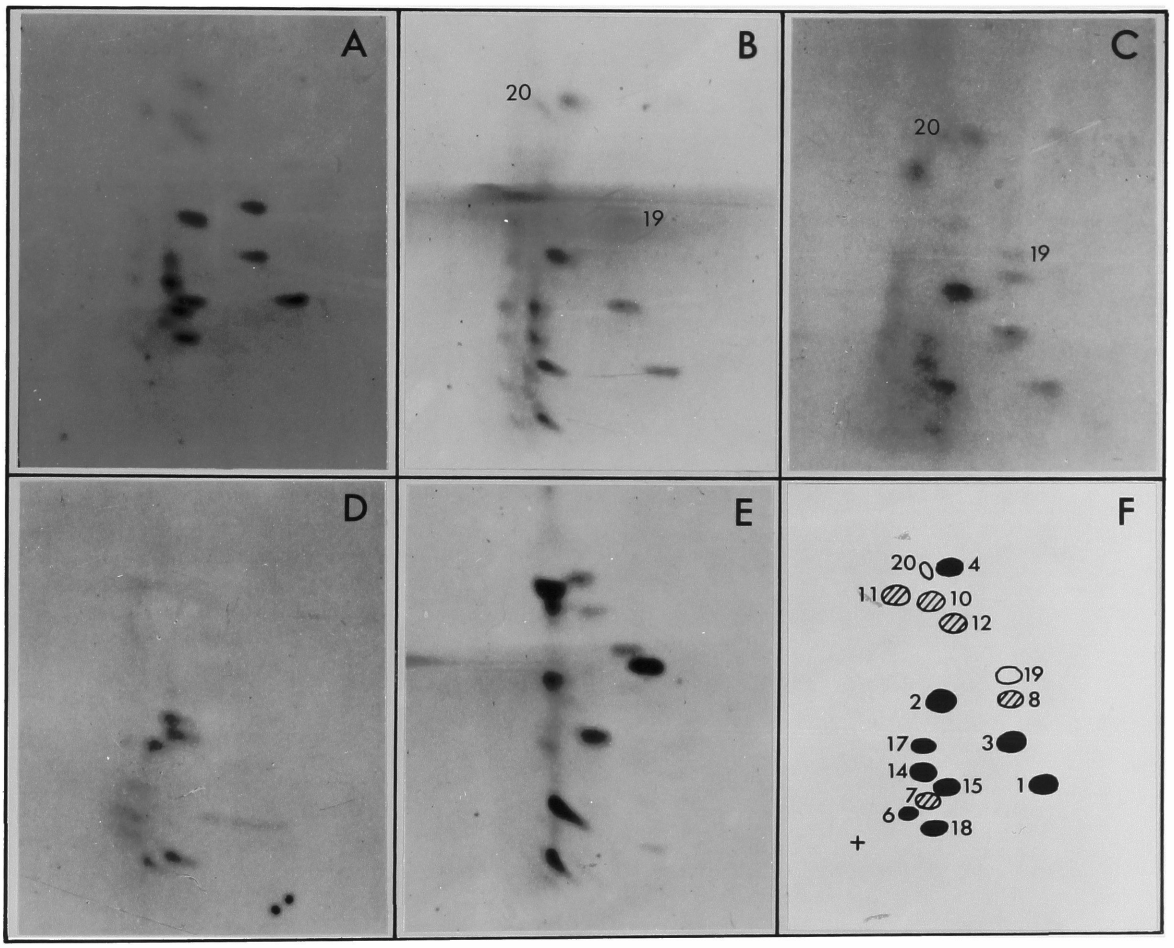
logical cross-reactivity between FSV P140 and the 98,000 dalton protein, which will be called NCP98 by analogy with NCP150 (the cellular homolog of the transforming protein of Abelson leukemia virus [142]).

Although no systematic comparison of the amount of NCP98 in bone marrows of chickens of different ages was made, no significant variation was detected in preparations obtained from 6 to 18 day old chickens.

NCP98 is structurally related to P140: In order to compare the structure of NCP98 and P140, we compared their tryptic peptides by two-dimensional fingerprint analysis. As seen in Fig. 2, the tryptic maps of NCP98 and P140 are closely related. The 62,000 dalton protein does not share any tryptic peptides with either fps-related protein. When the tryptic peptides of NCP98 and P140 were mixed prior to separation, 9 spots were found to be shared by the two proteins. Two spots are unique to NCP98 (see Fig. 2C). The gag precursor protein Pr76 of FAV is also shown.

Since we did not analyze a mixture of digests of P140 and Pr76 , it is difficult to determine unambiguously in our map which peptides in P140 are derived from the gag portion. However our map of P140 is in good agreement with the one reported by Beemon [6] who has identified the gag-derived spots as well as the FSV specific spots in P140. Although there is no direct proof that similarly migrating spots in the two maps are identical, it is likely that they are related.

Figure 2. Comparison of methionine-containing tryptic peptides of FSV P140, NCP98, 62,000 dalton protein and FAV Pr76. Tryptic digests of ^{35}S-methionine labeled proteins were spotted on cellulose plates and resolved by electrophoresis in the first dimension (from left to right) at pH 4.7, followed by ascending chromatography (from bottom to top).
(A) FSV P140; (B) NCP98; (C) FSV P140 and NCP98; (D) 62,000 dalton protein; (E) FAV Pr76; (F) schematic drawing of mixture of FSV P140 and NCP98. Most spots are numbered according to the notation of Beemon et al. [6]. Spots to the left of 14 and 17 were not taken into consideration, since they were not consistently seen and are believed to be the result of incomplete oxidation by performic acid. Solid circles: peptides common to FSV P140 and NCP98. Open circles: peptides unique to NCP98. Hatched circles: gag-derived peptides. +: origin.

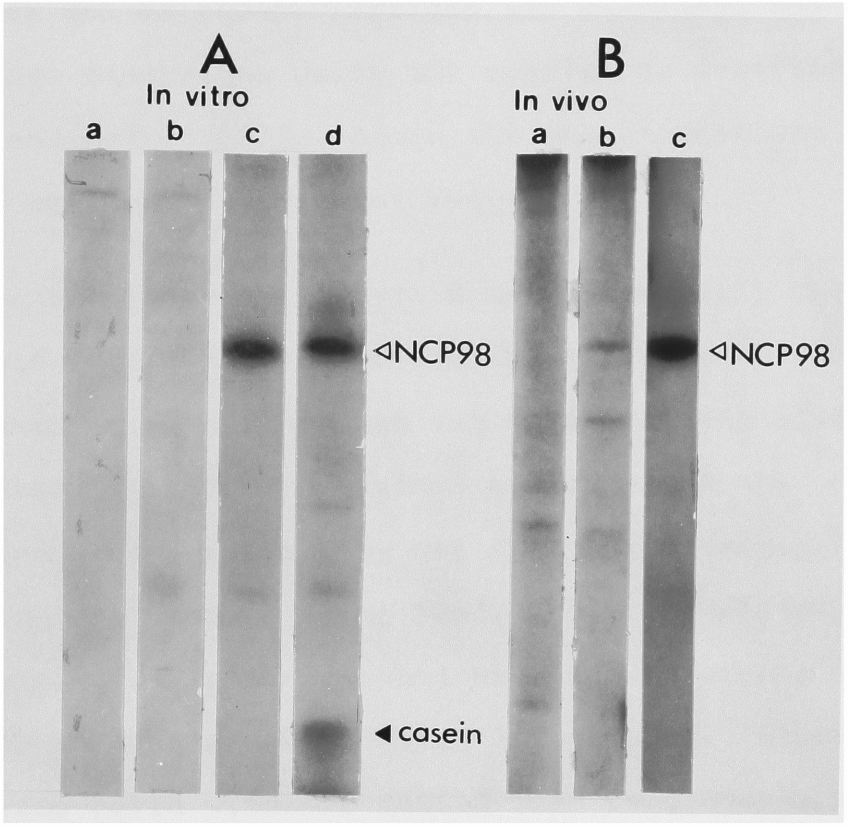

A
B
20
19
C
20
19
D
E
F
20
4
11
10
12
19
2
8
17
3
14
15
1
7
6
18
+

Therefore, we numbered most of our spots according to her notation. A summary of our findings is schematically drawn in Fig. 2, panel F.

In vitro protein kinase activity associated with NCP98: Since NCP98 is structurally related to P140, we tested whether NCP98, like P140, was also associated in vitro with a protein kinase activity. A bone marrow cell extract prepared from 10 day old chickens was immunoprecipitated with anti-fps antiserum and immune complexes were assayed for protein kinase activity. The products of the reaction were analyzed by SDS-PAGE. As seen in Fig. 3A (lane c), NCP98 itself is phosphorylated. When α-casein was externally added to the reaction mixture as a substrate, it also became phosphorylated. When anti-gag antiserum or TBR serum was used, no bands with the mobility of NCP98 could be detected, nor was any phosphorylation of α-casein observed (Fig. 3A, lane a, b).

Analysis of phosphoamino acids in NCP98 and α-casein labeled in vitro revealed that both proteins were phosphorylated exclusively at tyrosyl residues (Fig. 4D, E). Since NCP98 and P140 showed extensive homology in their tryptic peptide maps, we investigated whether in vitro phosphorylation occurred at the same sites in the two proteins. The in vitro ^{32}P-labeled proteins were digested with trypsin and the tryptic peptides were analyzed after separation in two dimensions. Both P140 and NCP98 yielded two main tryptic phosphopeptides after digestion that seemed to be identical in migration (Fig. 4A, B).

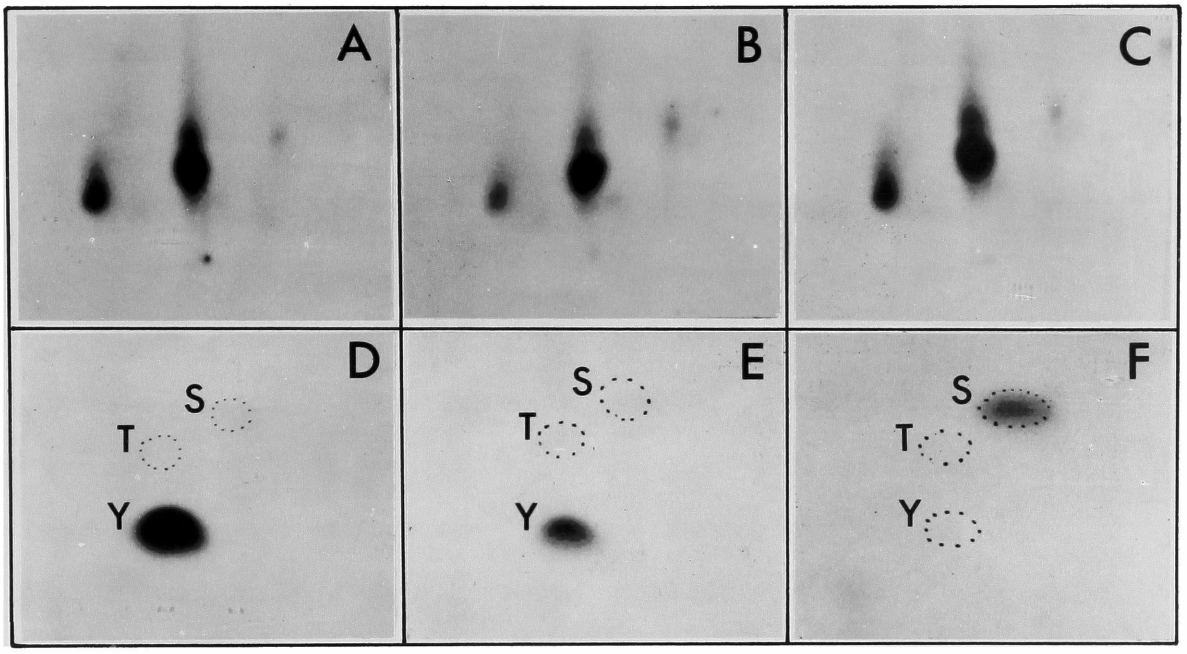
Figure 3. NCP98 is a phosphoprotein associated with protein kinase activity.
(A) An unlabeled cell extract prepared from uninfected chicken bone marrow cells was immunoprecipitated with the indicated antisera; the immune complex was resuspended in the kinase buffer (50 mM HEPES, 10 mM $MnCl_2$ and 10% glycerol [pH 7.4]) containing γ-^{32}P-ATP; the kinase reaction was carried out in the presence (lanes a, b, and d) or absence of α-casein (lane c), added as an exogenous substrate at 1 mg/ml. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and the gel was autoradiographed. Lane a, antivirion antiserum; lane b, TBR serum; lanes c and d, anti-*fps* antiserum.
(B) Uninfected chicken bone marrow cells were labeled with ^{32}P-orthophosphate and a cell extract was prepared and immunoprecipitated with non immune rat antiserum (lane a) or anti-*fps* antiserum (lanes b and c). Lane c, NCP98 labeled with ^{32}P in vivo was assayed for protein kinase activity in vitro as in lane c (A); only a small aliquot of the reaction product was loaded because of the difference in the radioactivity of labeled NCP98, obtained in the in vivo labeling and the in vitro protein kinase assay. Proteins were separated as in (A).


A
In vitro
a
b
c
d
◁NCP98
◀ casein
B
In vivo
a
b
c
◁NCP98

When the phosphopeptides of NCP98 and P140 were mixed together and analyzed under the same conditions, again the same two peptides could be detected (Fig. 4C), establishing that in vitro phosphorylation sites in NCP98 and P140 are identical. To ensure that comigration was not fortuitous, the phosphopeptides were further digested with the protease V8 of Staphylococus aureus. A new set of phosphopeptides was generated and analyzed in two dimensions using the conditions described by Patschinsky and Sefton [93]. Again the phosphopeptides of the two proteins comigrated (data not shown).

NCP98 is a phosphoprotein in vivo: Bone marrow cells obtained from 8 day old chickens were labeled for 2 h with ^{32}P-orthophosphate and a cell extract was made. Several aliquots were immunoprecipitated with various antisera and the precipitated proteins were separated by gel electrophoresis. When anti-fps serum was used, a faint band, which was absent when non-immune antiserum is used, could be detected with a mobility corresponding to that of NCP98 (Fig. 3B, lane b). Since labeling bone marrow cells with ^{32}P caused some cell death, we were concerned that it could result in a greater rate of dephosphorylation of NCP98 and hence affect the enzymatic activity associated with it. Therefore ^{32}P-labeled NCP98 was assayed after immunoprecipitation for its associated protein kinase activity. Only an aliquot of the in vitro protein kinase reaction was loaded on the gel (Fig. 3B, lane c).

Figure 4. Tryptic peptides and phosphoamino acid analysis of P140 and NCP98 kinase substrates in vivo and in vitro. Tryptic digests of ^{32}P-P140 (A), ^{32}P-NCP98 (B), and ^{32}P-P140 and ^{32}P-NCP98 (C) labeled in the in vitro protein kinase reaction, were separated in two dimensions. The tryptic peptides were subjected to electrophoresis in the horizontal dimension at pH 1.9, and subjected to ascending chromatography in the vertical dimension. The anode is to the right. Phosphoamino acid analysis was carried on ^{32}P-NCP98 autophosphorylated in vitro (D); ^{32}P-α-casein, added as a substrate to NCP98 in the in vitro protein kinase assay (E); and ^{32}P-NCP98 phosphorylated in vivo (F). Partial acid hydrolysates were separated in two dimensions: electrophoresis at pH 1.9 was carried out from left to right, and electrophoresis at pH 3.5 was run from bottom to top. S: phosphoserine; T: phosphothreonine; Y: phosphotyrosine.


A
B
C
D
S
T
Y
E
S
T
Y
F
S
T
Y

The increase in the radioactivity in the band of NCP98 indicates that under the conditions used, NCP98 labeled in vivo with ^{32}P is functionally active. Comigration of the in vivo labeled band (lane b) with the in vitro autophosphorylation product of NCP98 (lane c) further supports the conclusion that the band seen in Fig. 3B, lane b, is indeed NCP98. Analysis of phosphoamino acids of in vivo labeled NCP98 revealed only phosphoserine (Fig. 4F). This result is somewhat surprising because under the same conditions P140 is phosphorylated at both phosphoserine and phosphotyrosine [35,52,94].

The protein kinase activity associated with NCP98 is biochemically indistinguishable from that of P140: To characterize further the protein kinase activity associated with NCP98, we examined some of its biochemical properties. As seen in Table 1, the activity of NCP98 shows a strong preference for Mn^{2+} as a required divalent cation; Mg^{2+} on the other hand is a poor cation, and only 12% of the activity obtained with Mn^{2+} was detected. These results are in perfect agreement with those obtained for the protein kinase activity associated with P140 [35]. Wong et al. [144] synthesized a decapeptide corresponding to the tyrosine phosphorylation site of $p60^{src}$ [24,86,93,119]. When this peptide is present in the kinase assay mixture, it can inhibit the autophosphorylation of FSV P140, Y73 P90, and the phosphorylation of α-casein by $p60^{src}$.

Table 1. Divalent Cation Requirement and Inhibitor Sensitivity of NCP98 Associated Protein Kinase Activity

Cation		$pp60^{src}$ Inhibitor	NCP98		FSV P140	
Mn^{2+}	Mg^{2+}	Decapeptide	cpm	%	cpm	%
-	-	-	440	3	1,030	1
10 mM	-	-	13,750	100[a]	93,380	100[a]
-	10 mM	-	1,610	12	14,350	15
5 mM	5 mM	-	15,100	110	67,820	73
10 mM	-	-	12,900	100	93,700	100
10 mM	-	3.5 mM	1,625	13	11,260	12

Equal aliquots of a bone marrow cell extract or FSV-transformed CEF extract were immunoprecipitated with anti-fps. The immune complexes bound to Protein A-Sepharose were washed and resuspended in a kinase buffer containing 50 mM HEPES (pH 7.4), 10% glycerol, divalent cations as indicated and γ-^{32}P ATP at a final concentration of 4×10^{-7} M. When indicated, a decapeptide of $pp60^{src}$ (residues 415-424) corresponding to the in vitro tyrosine phosphorylation site (Wong et al., 1981) was added in the kinase buffer to a final concentration of 3.5 mM. ^{32}P-radioactivity was determined by counting gel slices containing NCP98 or P140 labeled during the in vitro kinase reaction. Percentage is expressed relative to the value obtained for NCP98 or P140 when Mn^{2+} is present at 10 mM (a).

Table 1 shows that the decapeptide brings about the same degree of inhibition (88%) of the autophosphorylation of NCP98 and P140, further demonstrating the similarity between the activities associated with the two proteins. As in the case of P140 [35], γ-^{32}P GTP did not serve as a ^{32}P donor molecule in the autophosphorylation of NCP98 (data not shown).

Distribution of NCP98 is tissue specific: Studies thus far described were conducted with extracts of bone marrow cells. We were interested to see whether, as it is the case with the level of c-fps mRNA [115], the expression of NCP98 varies in different tissues. For this analysis, we used the kinase assay as a means to quantitate NCP98, since it offered the advantage to be very sensitive even to small amount of NCP98, and was linear in the range of concentrations we were using. Several tissues from 9 day old chicks were homogenized, and aliquots of the various tissue extracts, each containing the same amount of protein, were immunoprecipitated with excess anti-fps serum. The kinase reaction was carried out for each sample and after protein separation by gel electrophoresis, radioactivity in excised gel bands containing ^{32}P-autophosphorylated NCP98 was determined. As shown in Table 2 the amount of NCP98 in different tissues agrees with the level of expression of c-fps mRNA reported by Shibuya et al. [115], with the exception of liver, in which the level of NCP98 expression was similar to that in lung (the copy number of c-fps mRNA found in lung being about 4 times that in liver).

Table 2. Amount of NCP98 in Different Tissues of Chickens

Tissue	Age of bird	cpm	%
Bone marrow	9 days	13,700	100
Liver	60 days	5,600	38
Liver	9 days	4,800	35
Lung	9 days	4,850	35
Spleen	9 days	3,700	27
Bursa	9 days	1,400	10
Thymus	9 days	1,370	10
AMV-infected myeloblasts	-	22,440	160
AEV-infected erythroblasts	-	3,000	21

Whole tissues were rinsed twice in ice cold isotonic buffer and homogenized in the modified NP40 buffer containing 0.1% 2-mercaptoethanol. The homogenates were clarified at 10,000 rpm for 10 min and aliquots containing the same amount of protein were immunoprecipitated with excess anti-fps. ^{32}P-radioactivity incorporated in NCP98 during the in vitro protein kinase reaction was determined. The percentage of radioactivity was expressed relative to the value of bone marrow cells.

We tried then to determine whether any particular type of cells in bone marrow was responsible for the synthesis of NCP98. For this purpose we made use of the in vivo specificity of infection of the acute leukemia viruses avian erythroblastosis virus (AEV) and avian myeloblastosis virus (AMV). AEV transforms chicken cells of erythroid lineage and AMV transforms myeloid cells present in bone marrow [44]. Two lines of bone marrow cells, transformed by AEV and AMV respectively, were lysed and the NCP98-protein kinase activity was determined as described above. AEV-infected cells expressed about one fifth of the activity found in total bone marrow cells, whereas myeloblasts infected with AMV showed an increased expression of NCP98 compared to total bone marrow cells (Table 2). The eight-fold difference observed in the expression of NCP98 in AMV-infected cells compared to the AEV-infected cells suggests that cells from the myeloid lineage rather than the erythroid lineage may contribute to the NCP98 expression detected in bone marrow tissue.

Since the level of c-fps expression in these cell lines might be influenced by AEV or AMV transformation, in collaboration with J. Samarut, we fractionated uninfected bone marrow cells by centrifugation on a BSA gradient [41]. This technique separates cells according to their density, and the more mature the cells are, the denser they are. NCP98 autophosphorylation and the number of cells of the granulocytic and erythrocytic lineage were determined for each fraction.

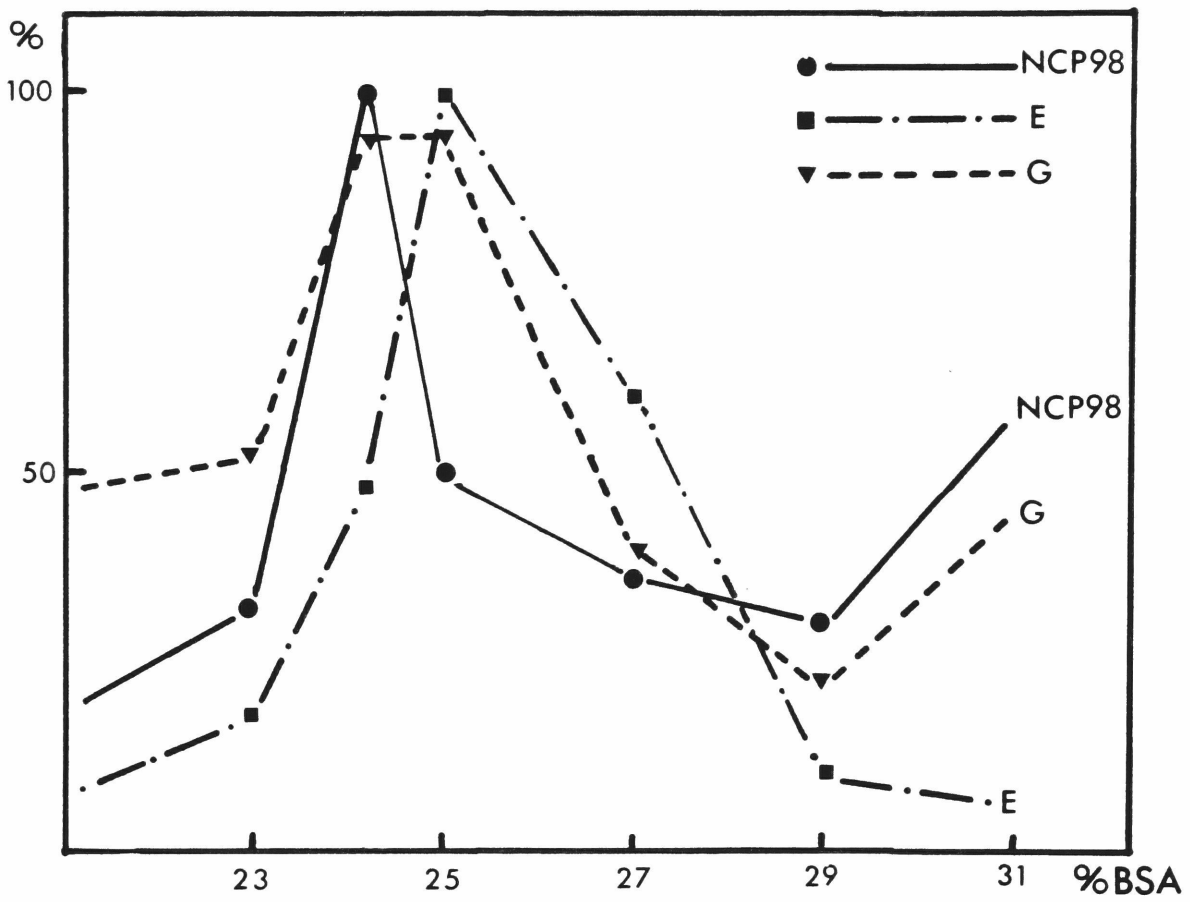


Figure 5. Profile of NCP98 activity versus granulocytic and erythrocytic cells distribution.
Ficoll-paque purified bone marrow cells were layered on successive 1.5 ml layers of BSA solutions increasing stepwise by 2% in concentration, from 21% to 31%. For each curve, the results are plotted as a percentage of the peak fraction either associated with the maximum in vitro kinase activity (NCP98), or with the maximum number of granulocytic (G) or erythrocytic (E) cells respectively. Cells of the erythrocytic or granulocytic lineages were identified by microscopic examination of each fraction. NCP98 activity was measured by assaying the protein kinase activity in the immune precipitate of total cell extracts of each fraction.

As can be seen on Fig. 5, the profile of NCP98 kinase activity follows the profile of sedimentation of cells of the granulocytic lineage. This result was further confirmed by immunocytolysis studies, in which bone marrow cells were incubated in the presence of complement and antibody directed against surface markers of erythroblasts. This resulted in the lysis of over 90% of the erythrocytic cells without affecting the recovery of NCP98 activity. Studies are currently underway to see whether NCP98 plays any role in the differentiation of early granuloblasts into mature granulocytes.

DISCUSSION.

Comparison of the cellular and viral fps gene product: The detection of several proteins, homologous to viral transforming proteins, in uninfected vertebrate cells has raised the question of their function(s) in these cells. These normal cell proteins share two characteristics that make them intriguing. First, there is a high degree of conservation of the amino acid sequence of a given class of c-onc gene product in several species that are distantly related in evolution [89,109]. Second, the expression of the c-onc proteins seems, to varying extents, to be tissue-specific [5,108,112,142]. These results suggest that these proteins are crucial at some stage of the life cycle of vertebrates and may play a role in differentiation and/or development.

Using a tumor regressor rat antiserum, anti-fps, we report the detection of a normal cell protein, NCP98, which is both structurally and functionally similar to the transforming protein of FSV. In addition to NCP98, our specific antiserum detected in uninfected bone marrow cells a protein of MW 62,000. By comparison of the tryptic peptides maps, no homology was found between NCP98 and the 62,000 dalton protein. The latter did not seem to be a phosphoprotein (since it was not detected in Fig. 3B lane b). No further characterization of this protein was undertaken.

The amino acid sequence for FSV P130 deduced from the nucleotide sequence obtained by Shibuya et al. [117] predicts a molecular weight for the fps-encoded sequence of 99,100 dal-

tons. Since we have identified a protein of MW 98,000 as the product of the c-fps gene, we conclude that most, if not all, of the c-fps coding sequences have been incorporated into the viral genome of FSV.

FSV and the avian sarcoma viruses PRCII-p and PRCIV share the fps sequence in their transforming genes [9,114], which presumably originated from cellular sequences present in uninfected chicken DNA. Breitman et al. [9] and Ghysdael et al. [42] estimated the size of the fps-specific sequences present in P170, the transforming protein coded by PRCII-p and PRCIV, to be about 120,000 daltons. The size determination was based either on the reactivity of P170 with monospecific antisera against the gag proteins p19 and p27, or on the size of the gag fragment removed after digestion of P170 with the viral protease p15. These estimates are slightly larger than the 98,000 daltons determined for NCP98. Some error in estimating the size of the gag sequences in P170 may be the cause for this observed difference. Alternatively, it is possible that the greater size of the fps-specific sequence in P170 is reflected in a longer C-terminal end of the protein due to the use of a different termination codon. In any case, this difference cannot be explained by the proposal that PRCII-p and PRCIV have acquired their fps sequence by recombining with another c-fps allele, because analysis of uninfected chicken DNA revealed only one c-fps locus per haploid genome [115].

Comparison of the tryptic maps obtained with FSV P140 and NCP98 revealed a high degree of similarity of the sequences in

the two proteins. Out of eleven spots resolved for NCP98, all but two had their counterpart in P140. That such a high homology in structure was reflected in identical properties of the protein kinase activity associated with NCP98 and FSV P140 was hardly surprising. Since NCP98 lacks gag sequences, we conclude that the gag region in P140 is not involved in its enzymatic activity, either directly as the enzymatic active center, or indirectly by binding a putative cellular protein kinase. Furthermore, the gag sequence present in P140 is not the site of the in vitro autophosphorylation, since NCP98 and P140 share the same tryptic phosphopeptides.

Although we only characterized the associated protein kinase of both proteins in vitro, we feel that the information is relevant to the in vivo properties of NCP98 and P140. Feldman et al. [35] have reported that out of three tryptic phosphopeptides detected in vivo in P140, two comigrate with the two tryptic phosphopeptides of P140 labeled in vitro. Using slightly different conditions for the separation, we also found two tryptic phosphopeptides in P140 labeled in vitro, and these exactly comigrate with the two in vitro phosphopeptides of NCP98. These in vitro labeled peptides are both phosphorylated at tyrosyl residues. However, we detected only phosphoserine in NCP98 labeled in vivo. The significance of this is not clear; it is possible that the short labeling time (2 h) was not sufficient to allow phosphorylation at tyrosine site(s) in bone marrow cells, if the phosphate group attached to tyrosine were to turn-over very slowly. However, longer labeling time

(4-6 h) did not result in phosphotyrosine labeling, and the incorporation of ^{32}P in NCP98 was still very low (data not shown). Our inability to detect phosphotyrosine may also reflect the possibility that this phosphoamino acid in NCP98 is present in vivo at only a small percentage of the level of phosphoserine. Phosphotyrosine may not be detectable, therefore, simply because the labeling of NCP98 with ^{32}P-orthophosphate was very inefficient in vivo. We are inclined to think that the absence of phosphotyrosine in NCP98 may reflect a technical problem rather than a relevant biological finding. We cannot rule out, however, that in vivo phosphorylation of NCP98 is under cellular regulation that is abolished in FSV-transformed cells thus allowing tyrosyl phosphorylation of P140 in vivo. Finally, under the conditions of labeling in which phosphoserine was detected, NCP98 still retained a functional associated protein kinase activity.

NCP98 expression: NCP98 was found most abundantly in bone marrow and in lesser amounts in liver, lung and spleen. This distribution basically agrees with the levels of c-fps mRNA expression determined by Shibuya et al. [115], except for liver. The good correlation between the NCP98 protein levels of expression and the c-fps mRNA quantitation in the different tissues suggests that the control of the level of NCP98 is primarily at the transcriptional level. In bone marrow cells, the expression of NCP98 may show considerable variation with the stage of development of the chicken. We have seen that the expression of NCP98 is slightly higher in AMV-infected myelob-

lasts and lower in AEV-infected erythroblasts, compared with total bone marrow cells. Moreover, our preliminary studies on uninfected bone marrow suggest that the expression of NCP98 varies with the origin of a cell type and possibly with the development stage of that cell type. Cells of the granulocytic lineage seemed to be the major cell type in bone marrow in which NCP98 was expressed; erythroblastic cells did not express NCP98 or only to a very small extent. We also observed that the expression of NCP98 in granuloblastic cells might correlate with the differentiation of early granuloblasts into mature granulocytes, but this last result needs to be confirmed in further expreiments. Concerning the possible relationship between fps expression and differentiation of hematopoietic cells, we do not know whether the enhanced expression of fps in granulocytes is a consequence of the differentiated state of these cells, or whether this enhancement is one of the events that triggers the differentiation of precursor hematopoietic cells into granuloid cells.

Nevertheless, the conclusion that is emerging from these experiments is that c-fps DNA is expressed in certain types of cells, and predominantly in cells belonging to the myeloid-differentiation pathway.

Activation of cellular oncogenes and transformation: We do not know whether NCP98 and FSV P140 share the same substrate specificity and the same subcellular localization in vivo. However the high degree of relatedness of the sequences of NCP98 and P140 is intriguing, since the latter is a transforming protein

coded for by a virus. Recently, D. Foster constructed a nondefective virus which had the src gene of RSV subgroup A replaced by fps-specific sequences derived from FSV (personal communication). This chimeric virus was able to transform chicken cells in tissue culture and coded for a fps protein of 95,000 daltons which lacked any gag sequences. This showed that the gag sequence present in P140 is not necessary for transformation. It remains to be seen, whether NCP98, when expressed at the same level as this 95,000 fps protein , can also cause transformation. Recent evidence suggests that this is the case for another cellular oncogene, c-myc, in chicken lymphomas induced by avian leukosis viruses (ALVs) . Analyses of DNA and RNA from ALV-induced lymphomas showed that in 85% of the tumors, integration of the ALV provirus, adjacent to c-myc, the cellular counterpart of the transforming gene of MC29 virus, caused enhanced expression of c-myc, leading to neoplastic transformation [54,95]. In the past few months, new links between the study of cellular oncogenes and chromosome abnormalities have been revealed in human tumors and mouse plasmacytomas (for review, see [63,104]). Translocation of the c-myc gene to the switch region of immunoglobulin heavy chain locus has been seen in Burkitt lymphoma and mouse plasmacytomas [13,23,26,85,113,127]. In these, the translocated c-myc gene is often found in opposite orientation relative to the heavy chain genes [23,113], and Shen-ong et al. have reported a c-myc RNA transcript about 400 bases shorter than normal [113]. Recent evidence indicates that c-myc transcription is elevated

about 10-20 fold in these cells as compared to normal B-cells [63], and that translocation of the c-myc gene to a new chromosome is not an absolute requirement for the increase seen in plasmacytoma or Burkitt lymphoma cells.

In addition, transformation of cells in tissue culture was achieved by transfecting cloned DNAs containing cellular sequences homologous to murine sarcoma viruses (MuSV) [27,92]. In these studies, cloned DNAs of the cellular sequences homologous to two viral transforming genes of the Moloney or Harvey strains of MuSV were transfected onto mouse cells. They were able to transform recipient cells only if these cellular sequences were ligated to viral long terminal repeats. Thus, the increase in the level of expression of these transfected cellular sequences appeared to be responsible for transformation. Lastly, in several instances the tumorigenicity of DNA from human tumors has been traced to the activation of one of the proto-oncogenes homologous to the viral transforming genes, v-Ha- or v-Ki-ras, of the Harvey or Kirsten strain of MuSV, respectively, suggesting that a change in the function of either of these can be a step in the formation of cancer in several human organs [15,43,101,124]. In bladder carcinoma, the tumorigenicity of DNA seems to be due, not to a change in the level of expression of the oncogene, but to a point mutation in the sequence of the protooncogene that causes the conversion of a glycine to a valine residue [101,124].

In summary, three types of activation of cellular oncogene have been described.

(i). An increase in the level of expression of cellular oncogenes has been shown to induce lymphoma in chicken [54,95] or to cause transformation of mouse fibroblasts in tissue culture [27,92], and has been postulated as one of the steps in the process of oncogenesis in murine and human B-cells lymphoma [63].

(ii). Gene rearrangement of cellular oncogenes has been observed in human neoplasms and particularly in the hematopoietic system [104]. Whether this rearrangement simply leads to an increase of the amount of the oncogene or also to a structural modification of this oncogene remains still unanswered.

(iii). Finally, activation of the ras gene family by mutation has been implicated as a step in tumorigenicity in humans; there also, the interpretation of the results must remain guarded, since transfection of the cell line NIH 3T3 has some inherent problem; the transforming capacity of the activated ras gene should be tested on other cells, before assessing the significance of the above-mentioned result.

It becomes apparent, however, that no theory of oncogenesis can ignore the central importance of cellular oncogenes in the process of transformation.

- III -

REVERTANTS OF RAT CELLS TRANSFORMED BY FUJINAMI SARCOMA VIRUS.

One useful approach in understanding the various factors involved in transformation by retroviruses has been the isolation of clones of transformed cells which have reverted to a normal phenotype, and to study the underlying mechanisms that led to that reversion. Theoretically, reversion can be caused either by a mutation within the viral genome, or by a mutation in cellular sequences. As a result, any step in transcription, RNA processing, translation and posttranslational modification of the transforming protein can be affected. In fact four types of revertants have been observed; usually, reversion results from the loss of the provirus [3,30], from a transcriptional block of the provirus [16,97,98], or from the presence of a mutation in the provirus responsible for the synthesis of a non-conditional, transformation defective protein [2,4,133]. Less frequently, alterations in cell functions required for both morphological transformation and tumorigenicity have been implied as a possible cause of reversion to a normal morphology [67, Bassin personal communication].

Studies involving the isolation of revertants require the cloning of single cells, and the maintenance of these clones over several generation to allow the biochemical characterization of homogenous population of cells. In the case of avian retroviruses, this requirement precludes the use of chicken cells, since, unlike in mammalian cells, there is no esta-

blished cell line. However, in some cases, mammalian cell lines have been successfully infected with avian retroviruses, and have been used in the isolation of transformed and reverted cells. Mammalian cells offer the additional advantages that they do not contain any endogenous avian retroviral sequences in their DNA, are non-productively infected with these viruses, and contain rarely more than one copy of viral DNA integrated in their chromosome. In the past, various rodent cells transformed by Rous sarcoma virus (RSV), B77, or avian erythroblastosis virus (AEV) have been obtained, allowing the isolation and characterization of reverted cells which were originally transformed by these viruses [8,16,29,64,88,98,132].

Fujinami sarcoma virus (FSV) proved to be useful in this kind of study because it was infectious for rat cells in tissue culture, although the efficiency of infection was reduced a thousand fold as compared to chicken cells [77]. These transformed cells did not produce viral particles and contained only one FSV provirus in their chromosome, which coded for P130, the only viral protein detected in these cells. We isolated various types of revertants and analyzed in each case the cause and possible mechanisms that led to the reversion.

RESULTS

Isolation of revertant clones: Rat fibroblasts 3Y1 were infected with the strain of FSV which codes for P130; 12 foci were isolated after 2-3 weeks and transformed cells in each focus were cloned. In this process, cells derived from focus #5 yielded two clones, 5G and 5H, which showed intermediate levels of transformation, These clones were treated with either colcemid or FUdR, a procedure which was described to improve the frequency of isolation of revertant cells [88]. Treated cultures were subcloned, and ten independent revertant clones were isolated from 5G and 5H, four of which were further characterized. As indicated in Fig. 1, all the revertants derived from focus #5 (including the six clones which were not extensively studied) yielded spontaneous retransformants with a frequency of about 10^{-6} to 10^{-7}. Cells obtained from focus #9 and #12 did not give rise spontaneously to partial revertants. Thus, selected clones, transformants 9G and 12A, were treated with hydroxyurea, a procedure known to select for quiescent cells in cultures maintained at high density [133]. This selection was repeated again twice, allowing time in between for surviving cells to reach confluence. The cells were then treated with FUdR and plated at low density to allow a first rapid screening of colonies. After screening an average of 5-10,000 cells from each clone, we isolated one colony from 9G and four from 12A which showed normal morphologies.

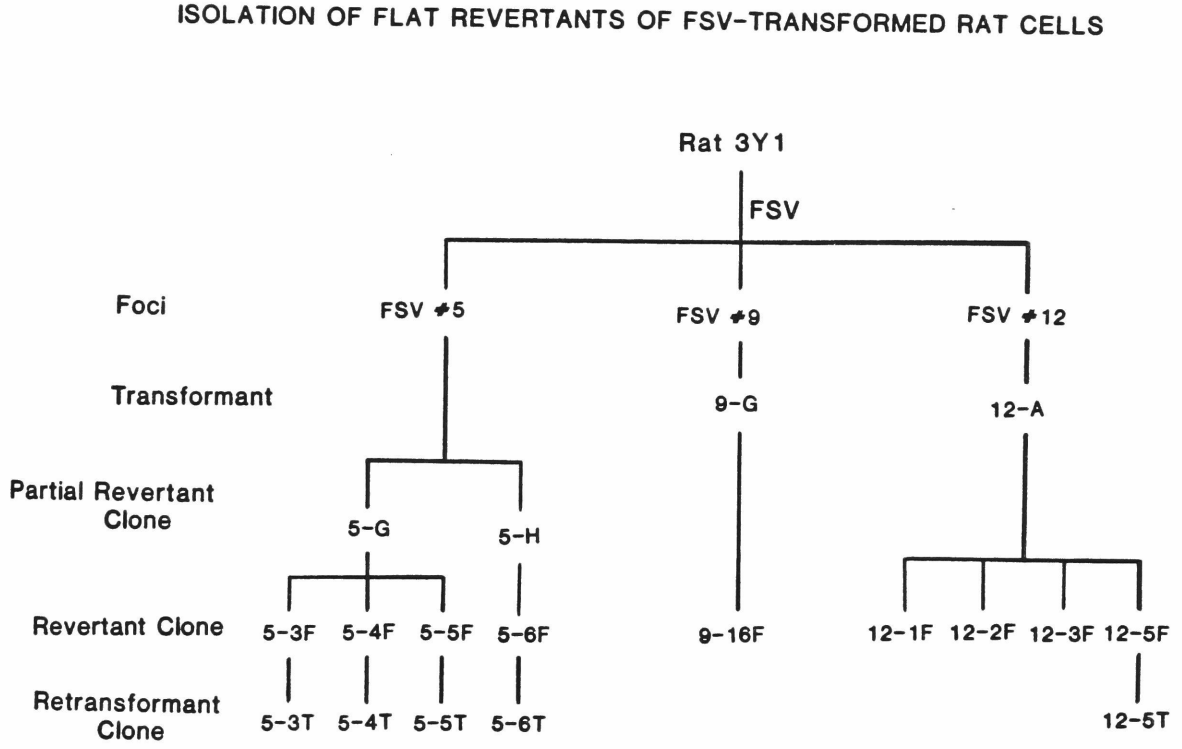
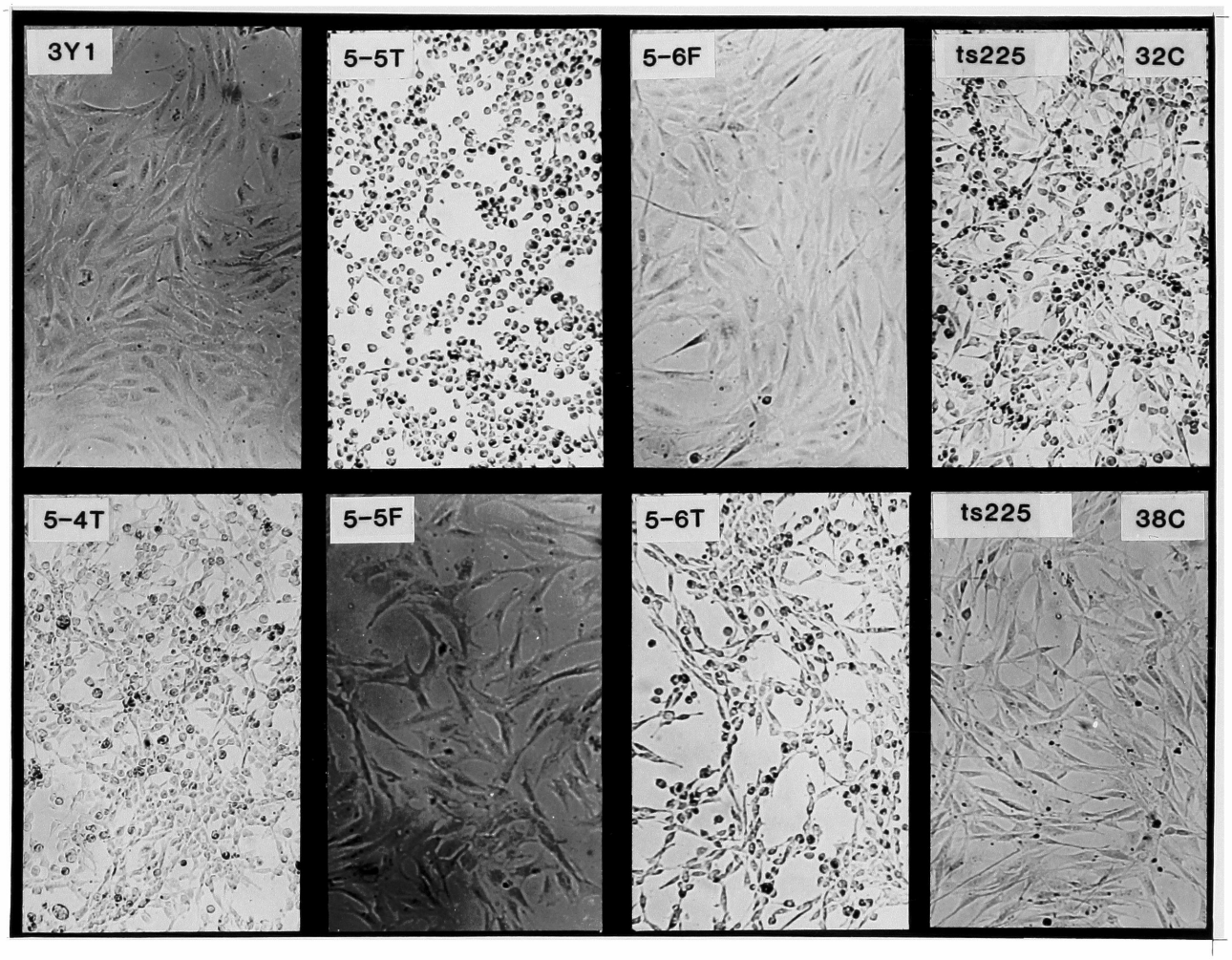


Figure 1. Schematic representation of the derivation of various revertants and retransformants.
Every step after the isolation of foci #5, #9, and #12 was preceded by cell cloning. The nomenclature of the isolated clones is as followed: The first numeral refers to the original focus from which a clone was derived.
Parental clone: for each single clone obtained after the first round of cloning, this numeral is followed by a capital letter (different from F or T). Each original clone was then treated with various drugs (as described in the text), and surviving cells were subcloned.
Subclones: the capital letter of the parental clone has been replaced by a numeral followed by the letter F (for flat morphology) in the revertants. When the revertants retransformed spontaneously, the letter T (for transformation) was used instead of F, in the cloned retransformants.

Each colony was then subcloned, and representative flat subclones were selected for further experiments. Among these revertants, only one (clone 12-5F) retransformed, with a similar frequency as that observed in the focus #5 series; the other four revertants maintained their flat morphology even after three months of subculturing. Fig. 2 shows the typical morphology of revertants derived from focus #5, with their spontaneous retransformants. Revertants 5-5F and -6F displayed a morphology identical to that of uninfected 3Y1 cells. Cells were flat, non refractile, and showed a high degree of contact inhibition at confluence. On the other hand, the morphology of retransformants 5-4T, -5T and -6T was radically different. Cells were round or spindle-form, highly refractile and were characterized by a three to five fold increase in their saturation density. For comparison, 3Y1 cultures infected with the ts mutant FSV NY225 were included. Cells maintained at 32 C (the permissive temperature) had a morphology identical to that of the retransformants, whereas cells at 38 C (the non-permissive temperature) had adopted a normal-like appearance.

Reversion to a normal phenotype of cells transformed by retroviruses has been ascribed to several mechanisms [16,29,67,97,132]. To distinguish among these, a biochemical analysis of the reverted cells was performed at the DNA, RNA and protein levels. Fig. 3 illustrates the results of the DNA analysis of various transformants, revertants and retransformants.

Figure 2. Morphology of FSV-infected rat fibroblasts in revertants, retransformants and ts mutant-infected cultures. 3Y1: uninfected rat fibroblasts; 5-5F and 5-6F: revertant clones of 3Y1 cells originally transformed by FSV; 5-4T, 5-5T and 5-6T: spontaneous retransformants of reverted clones 5-4F, 5-5F and 5-6F; 225: 3Y1 cells infected with FSV ts mutant NY225, and maintained at the permissive (32 C) or non permissive (38 C) temperature.


3Y1
5-5T
5-6F
ts225
32C
5-4T
5-5F
5-6T
ts225
38C

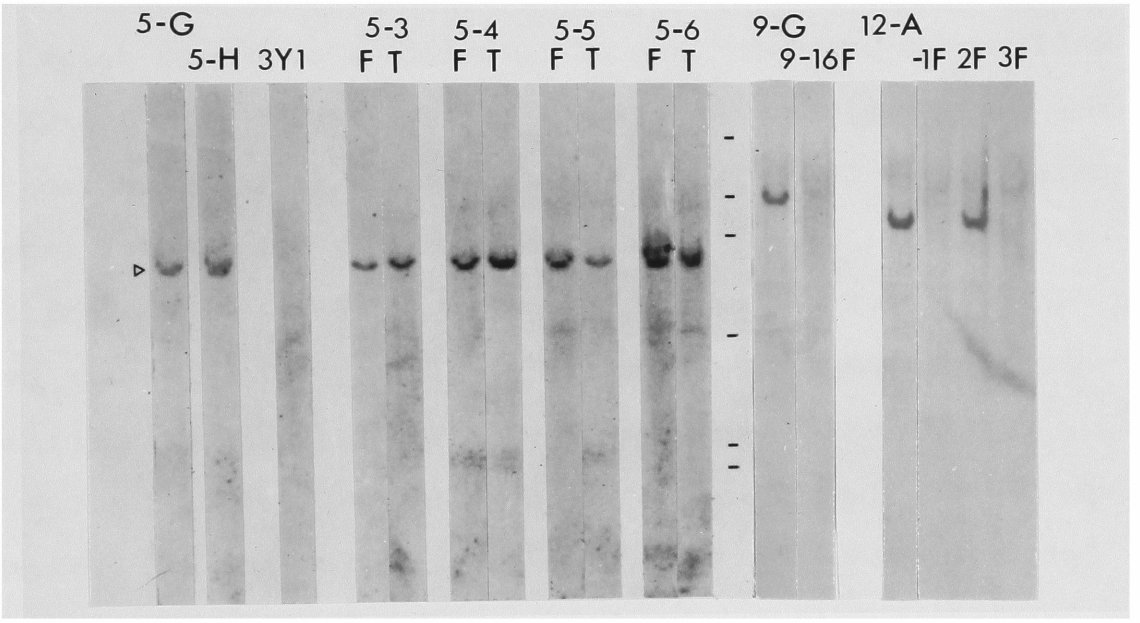
High molecular weight DNA from each cell clone was digested with Hind III, and the digestion product was run on an agarose gel. After transfer to nitrocellulose paper, the DNA fragments were hybridized to a probe representing the whole FSV provirus (FSV_{rep}). Since Hind III does not cut inside the FSV provirus [116], each hybridized fragment will be specific for a given integration site of FSV in the host chromosome. This analysis revealed the following:

First, parental clones 5H and 5G had a Hind III restriction fragment distinct from that of the parental clones 9G or 12A, indicating that each original focus that had been selected was the result of an independent infectious event.

Second, three revertant clones (clones 9-16F, 12-1F and 12-3F) had lost the entire FSV provirus. We called this type of revertants, class I revertants. Since they represented a simple case of reversion, they were not studied further, but each of them was susceptible to transformation after superinfection with FSV. Third, digestion of the DNA of clone 12-2F with Hind III resulted in the same fragment as the one found for the parental transformant, clone 12A. Furthermore, no gross rearrangement of 12-2F proviral DNA could be detected after digestion with Bam Hl, which cuts three times in the provirus.

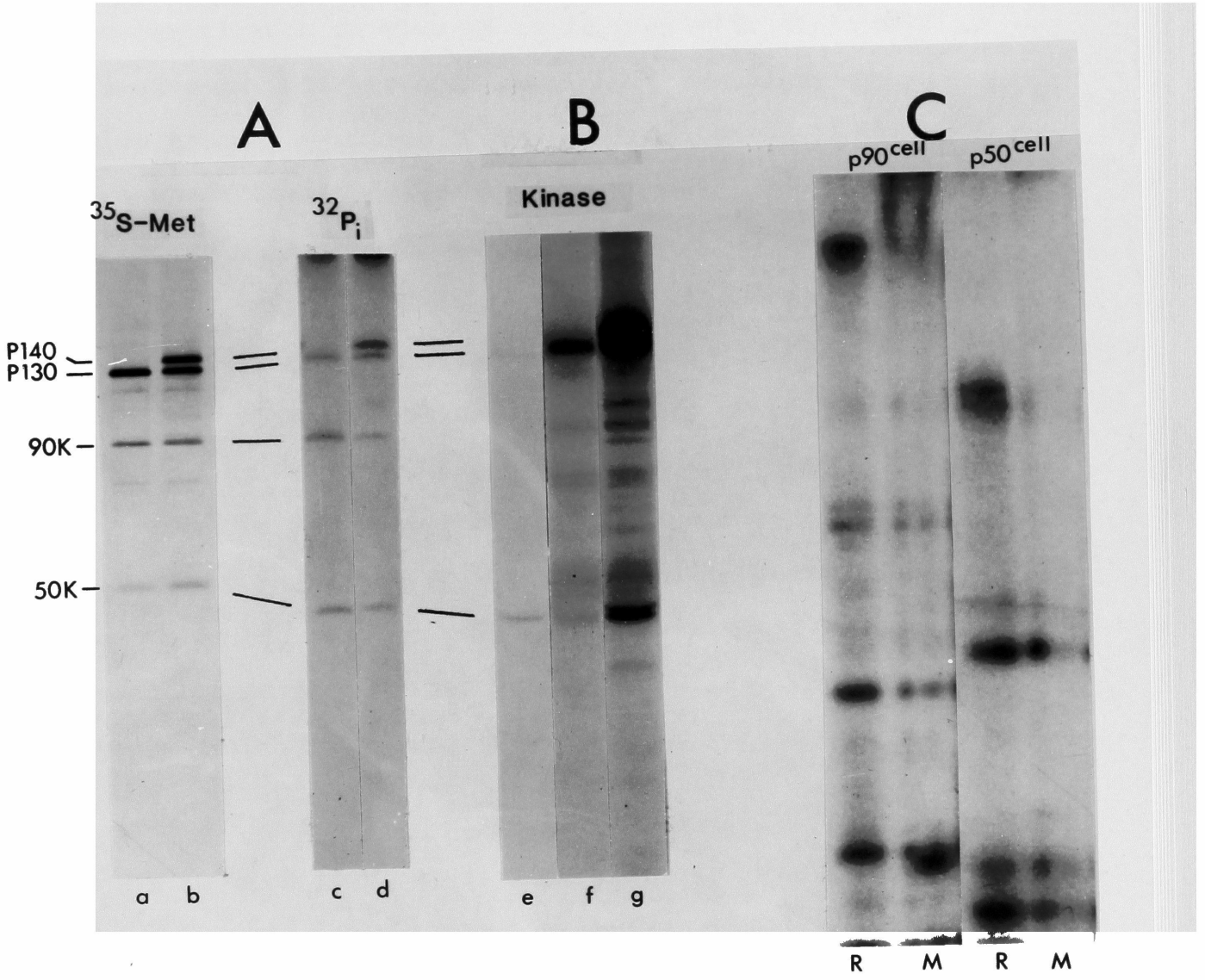
Finally, clones 5-3F, -4F, -5F and -6F shared with their spontaneous retransformants, 5-3T, -4T, -5T, -6T respectively, the same Hind III fragment characteristic of clones 5H and 5G. Proviral DNAs of these clones showed no rearrangement as judged by Bam Hl digestion.

Figure 3. FSV-specific DNA restriction fragments in FSV-infected clones.
High molecular weight DNA from various clones was digested with the restriction enzyme Hind III. DNA fragments were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with FSV_{rep} probe. The identity of each clone is indicated above each lane. F (flat morphology) denotes revertants and T (transformed morphology) denotes retransformants. The lineage of these clones can be seen in Fig. 1. Results obtained with 3Y1 cells and clones of the #5 series and those obtained with clones of the #9 and #12 series were derived from two different experiments. Markers obtained after digestion of λ phage with Hind III are indicated (23.3 kb; 9.5kb; 6.4 kb; 4.2 kb; 2.2 kb; 1.8 kb).


5-G
5-H
3Y1
5-3
F T
5-4
F T
5-5
F T
5-6
F T
9-G
9-16F
12-A
-1F
2F
3F

Class II revertant: Among the revertants that had kept a copy of the FSV provirus, clone 12-2F had the unique property of not giving rise to spontaneous retransformants. To examine whether a viral protein was expressed in this clone, a cell extract of 12-2F labeled in vivo with ^{35}S-methionine or ^{32}P-orthophosphate was immunoprecipitated with anti-fps (Fig. 4). Lanes a and c show that a protein of 130,000 daltons (or slightly less) was detected in clone 12-2F. This protein was expressed at similar level as that of P130 wild-type in a FSV-transformed 3Y1 culture, which served as a control. It was, however, enzymatically inactive when it was assayed for kinase activity in the immune complex (lane e), whereas wild-type P130 was readily autophosphorylated under the same conditions (lane f). Thus, the viral protein expressed in 12-2F was a mutated protein which lacked kinase activity, and was called 12-2F P130. Another interesting aspect of 12-2F P130 was its association in vivo with a 50,000 and a 90,000 dalton protein. These two proteins were identified as the cellular proteins, 50K and 90K, previously shown by Brugge and others to preferentially bind deficient protein kinases of avian sarcoma viruses [1,11,72,91]. The identities of these proteins were confirmed by comparing their phosphopeptides after partial digestion with the protease V8 to those of bona fide 50K and 90K isolated from mouse cells infected with a ts mutant of RSV, and which were a gift from J. Brugge.

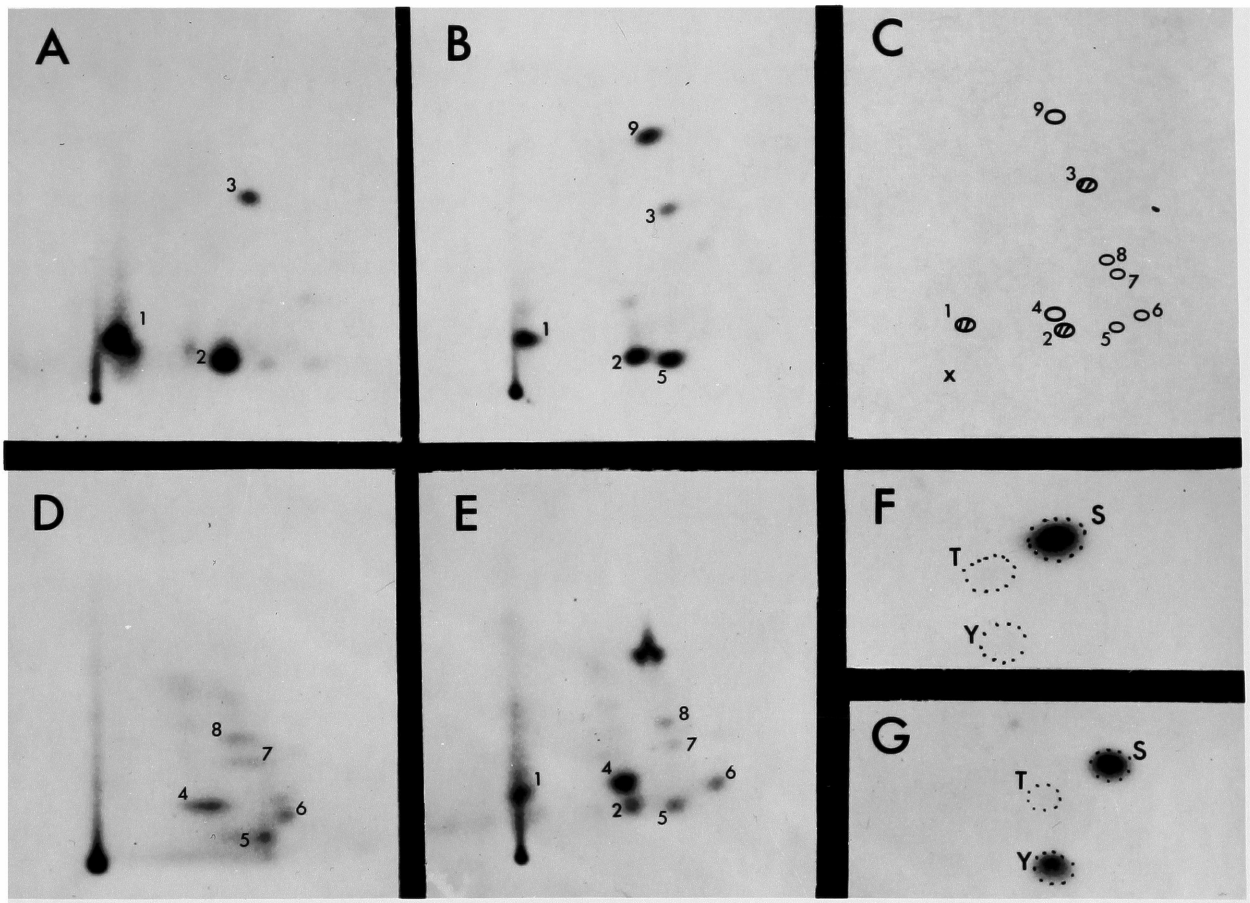
Figure 4. Analysis of the FSV viral protein complex in clone 12-2F.
(A) Cell extracts of cultures labeled with ^{35}S-methionine (lanes a, b) or ^{32}P-orthophosphate (lanes c, d) were immunoprecipitated with anti-*fps*, and the proteins separated by SDS-polyacrylamide gel electrophoresis (8.5%). Lanes a, c: clone 12-2F; lanes b, d: clone 12-2ST1.
(B) Cell extracts of unlabeled cultures were assayed for protein kinase activity after immunoprecipitation with anti-*fps*. The immune complex was resuspended in kinase buffer containing 2×10^{-7} M γ-^{32}P-ATP, and the reaction was stopped after a 15 min incubation at 25 C. Proteins were separated as in (A). Lane e: clone 12-2F; lane f: wild-type FSV-infected clone; lane g: clone 12-ST1. (C) Partial V8 digestion of ^{32}P-labeled 90K and 50K. Gel slices containing the labeled proteins were treated with 75 ng of V8 protease each, and the the digestion products were separated on a 12.5% polyacrylamide gel. R_{90}: 90K protein isolated from clone 12-2F (rat cells); M_{90}: 90K isolated from mouse cells; R_{50}: 50K isolated from clone 12-2F (rat cells); M_{50}: 50K from mouse cells.


A
B
C
35S-Met
32Pi
Kinase
p90cell
p50cell
P140
P130
90K
50K
a
b
c
d
e
f
g
R
M
R
M

As shown in Fig. 4, the V8 peptides of the 90K and 50K proteins which coprecipitate with 12-2F P130 were identical to those of the murine 90K and 50K complexed with the ts mutant-encoded $p60^{src}$. The association in vivo of 12-2F P130 with these two proteins was also an indirect confirmation of the transformation defective character of the former protein, since 90K and 50K bind more specifically defective tyrosine kinases. Wild-type P130 , on the other hand, was not complexed to any appreciable extent with 90K and 50K in the cell. Like Class I revertants, clone 12-2F can be retransformed by superinfection with FSV. In this instance, we used the strain of FSV which codes for P140 to be able to distinguish the gene product of the resident provirus in 12-2F from that of the superinfecting FSV. Lanes b and d in Fig. 4 show that 12-2ST1, the superinfected clone which had been labeled in the same conditions as 12-2F, in addition to 12-2F P130, synthesized P140, the transforming protein of the superinfecting FSV virus.

Phosphorylation sites of 12-2F P130 : In clone 12-2F, P130 is a phosphoprotein in vivo (Fig. 4, lane c) which is phosphorylated only on serine residues (panel 5F). However, P130, in the superinfected clone 12-2ST1, is phosphorylated both on serine and tyrosine residues (panel 5G). Thus, the two phosphorylated forms of P130 were useful in mapping the in vivo tyrosyl phosphorylation sites of 12-2F P130. The tryptic phosphopeptides of both forms of P130 labeled in vivo with ^{32}P-orthophosphate were separated in two dimensions and they were compared to those obtained from wild-type P130 and P140.

Figure 5. Tryptic phosphopeptides and phosphoamino acid analysis of 12-2F P130, wild-type P140 and P130 labeled in vivo.
Tryptic digests of ^{32}P-P140 (A), wild-type ^{32}P-P130 (B), 12-2F ^{32}P-P130 and 12-2ST1 ^{32}P-P130 labeled in vivo were separated in two dimensions. The tryptic peptides were subjected to electrophoresis in the horizontal dimension at pH 1.9, and subjected to ascending chromatography in the vertical dimension. The anode is to the left. (C) Schematic diagram of the various phosphopeptides found in A-E. Hatched circles represent the conserved phosphotyrosine-containing tryptic peptides in wild-type P140 and P130, and in 12-2ST1 P130.
Phosphoamino acid composition of 12-2F P130 (F) and 12-2ST1 (G). Partial hydrolysates were separated in two dimensions; electrophoresis at pH 1.9 was carried out from left to right followed by electrophoresis at pH 3.5 from bottom to top. S: phosphoserine; T: phosphothreonine; Y: phosphotyrosine.


A
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C
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F
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G
S
T
Y

Panel 5D shows the tryptic phosphopeptides of P130 isolated from 12-2F; all of them contained phosphoserine, since it was the only phosphoamino acid that was detected in this form of P130 (panel 5F). Panel 5E shows the same analysis for the form of P130 isolated from clone 12-2ST1. In addition to the peptides shown in panel D, there were three new peptides (spots 1, 2, and very likely 3) which were likely to contain phosphotyrosine, since in this case, phosphotyrosine and phosphoserine were detected in P130 (panel 5G). Panels A and B show the results obtained for wild-type P140 and P130 , respectively. Spots 1, 2, and 3, which were found in P130 isolated from 12-2ST1, were conserved both in wild-type P140 and P130. These spots have been shown in the case of P140 to contain only phosphotyrosine (R.A. Feldman, personal communication). Panel C is a schematic diagram of the different phosphopeptides observed for wild-type P140 and P130, and P130 isolated from 12-2F and 12-2ST1. Since spots 1 and 2 are also the site of in vitro phosphorylation of P130 (unpublished results), these results argue against the possibility that the failure of 12-2F P130 to autophosphorylate in vitro would be due to an impaired phosphate-accepting capacity of this protein, rather than to a loss of enzyme activity. Moreover, when an extract of 12-2ST1 was assayed for kinase activity after immunoprecipitation with anti-fps, both P140 and 12-2F P130 were phosphorylated, on tyrosine only (Fig. 4, lane g). This suggests that 12-2F P130 can serve as a substrate of P140, both in vivo and in vitro.

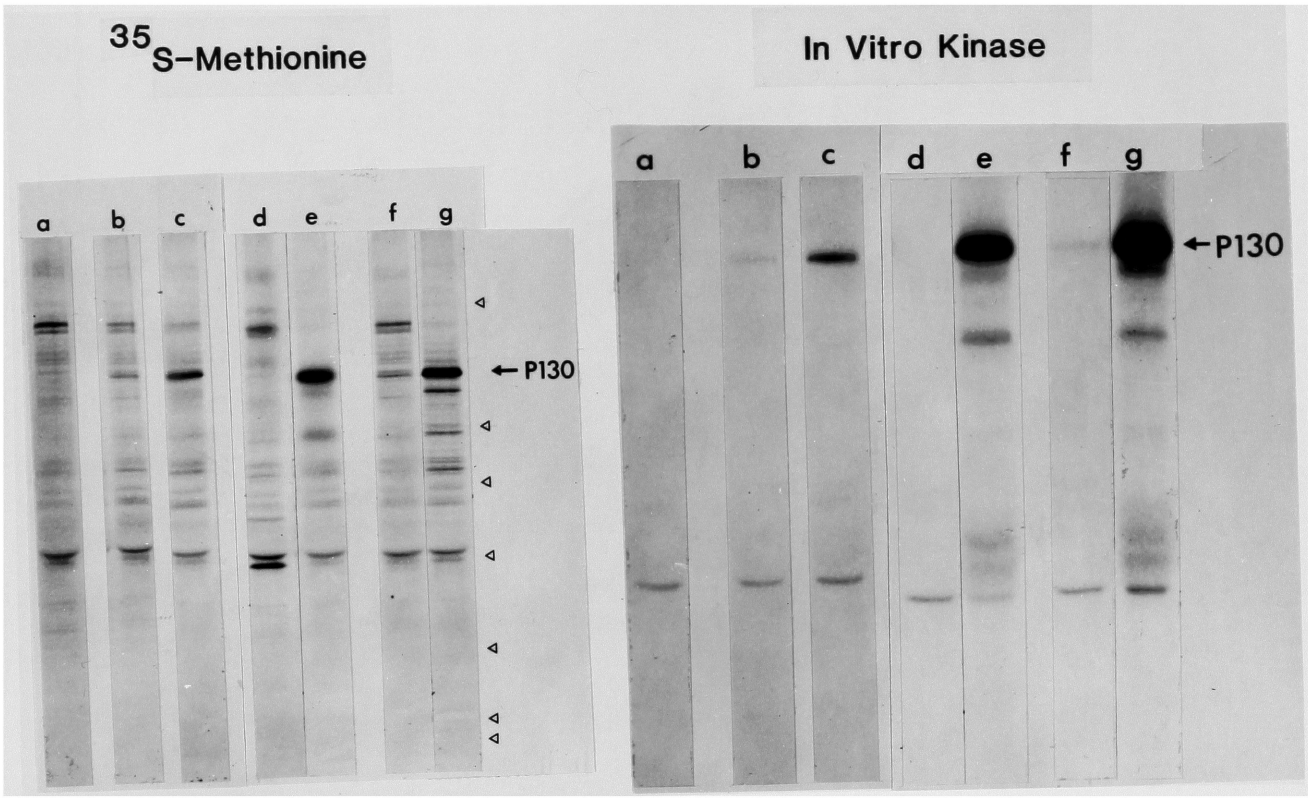
Therefore, a mutation (most probably a small deletion) of

FSV proviral DNA resulted in the synthesis of a defective transforming protein in clone 12-2F, and we have classified this type of revertant as class II revertant.

Class III revertants: A number of revertants had the ability to retransform spontaneously with a frequency of 10^{-6} to 10^{-7}. FSV proviral DNA of these flat revertants and their retransformants shared the same integration site and was not rearranged. To examine whether such revertants had a functional provirus which was not allowed to be expressed, we labeled the revertant clones and their retransformants with ^{35}S-methionine. Cell extracts were immunoprecipitated with antivirion antiserum to test for the presence of P130. Fig. 6 shows the results of this analysis. In lane a, immunoprecipitation of uninfected 3Y1 cells is shown as a control. Lanes b and c illustrate the case of the parental clones 5G and 5H, from which the revertants were derived; they show low or intermediate levels of P130. The revertant clones 5-5F and -6F (lanes d, f) expressed no or very little P130, whereas the retransformants 5-5T and -6T (lanes e, g) showed high levels of P130. Similar results were obtained when the level of P130 synthesis was measured by the in vitro kinase assay, (see kinase panel).

We then checked by liquid hybridization the amount of v-fps mRNA in the revertants and in the retransformants. Total RNA was extracted and hybridized to a labeled $cDNA_{fps}$ probe to various C_rt values.

Figure 6. P130 levels in clones derived from partial transformants 5G and 5H.
P130 was detected either after immunoprecipitation of ^{35}S-methionine labeled cell extracts with antivirion antiserum (^{35}S-methionine panel) or after in vitro autophosphorylation of P130 in the immune complex obtained after immunoprecipitation of unlabeled cell extracts with antivirion antiserum (in vitro kinase panel). Proteins were separated as in figure 4A and 4B. Lane a: 3Y1 cells; b: clone 5G; c: clone 5H; d: clone 5-5F; e: clone 5-5T; f: clone 5-6F; g: clone 5-6T.


35S-Methionine
In Vitro Kinase
a
b
c
d
e
f
g
P130
P130

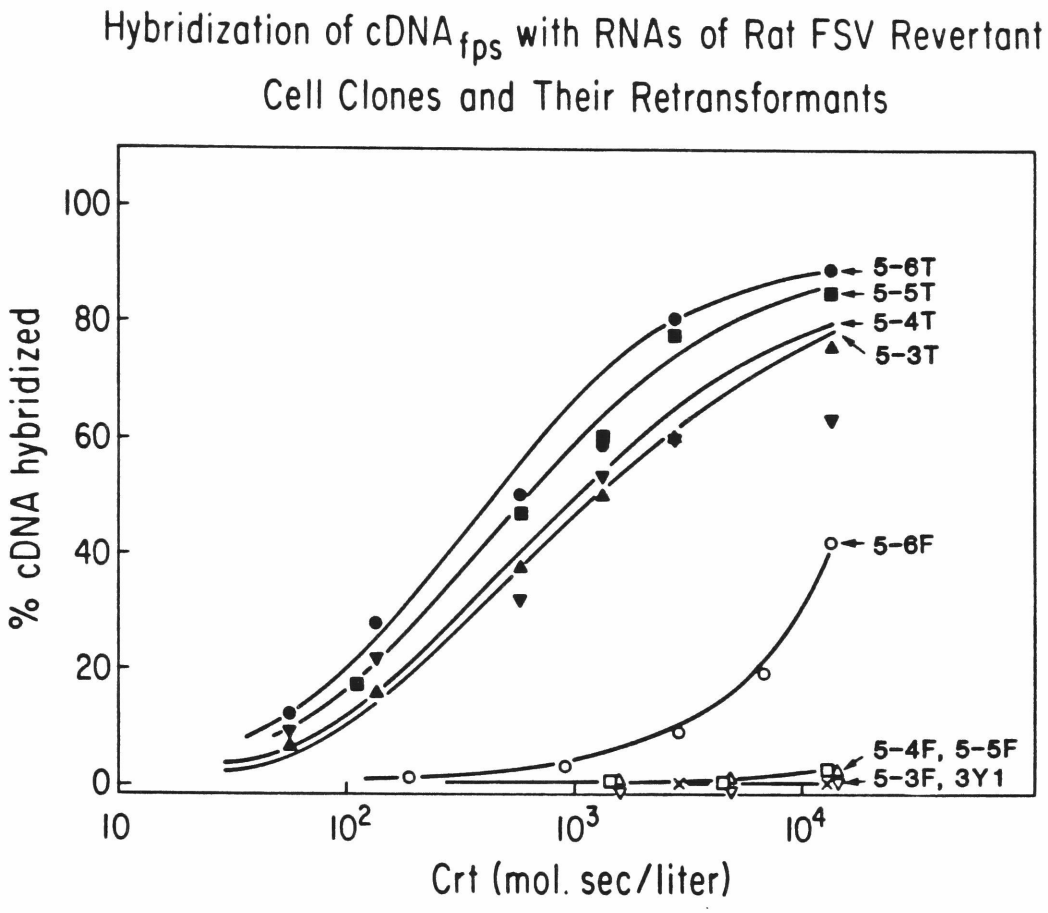
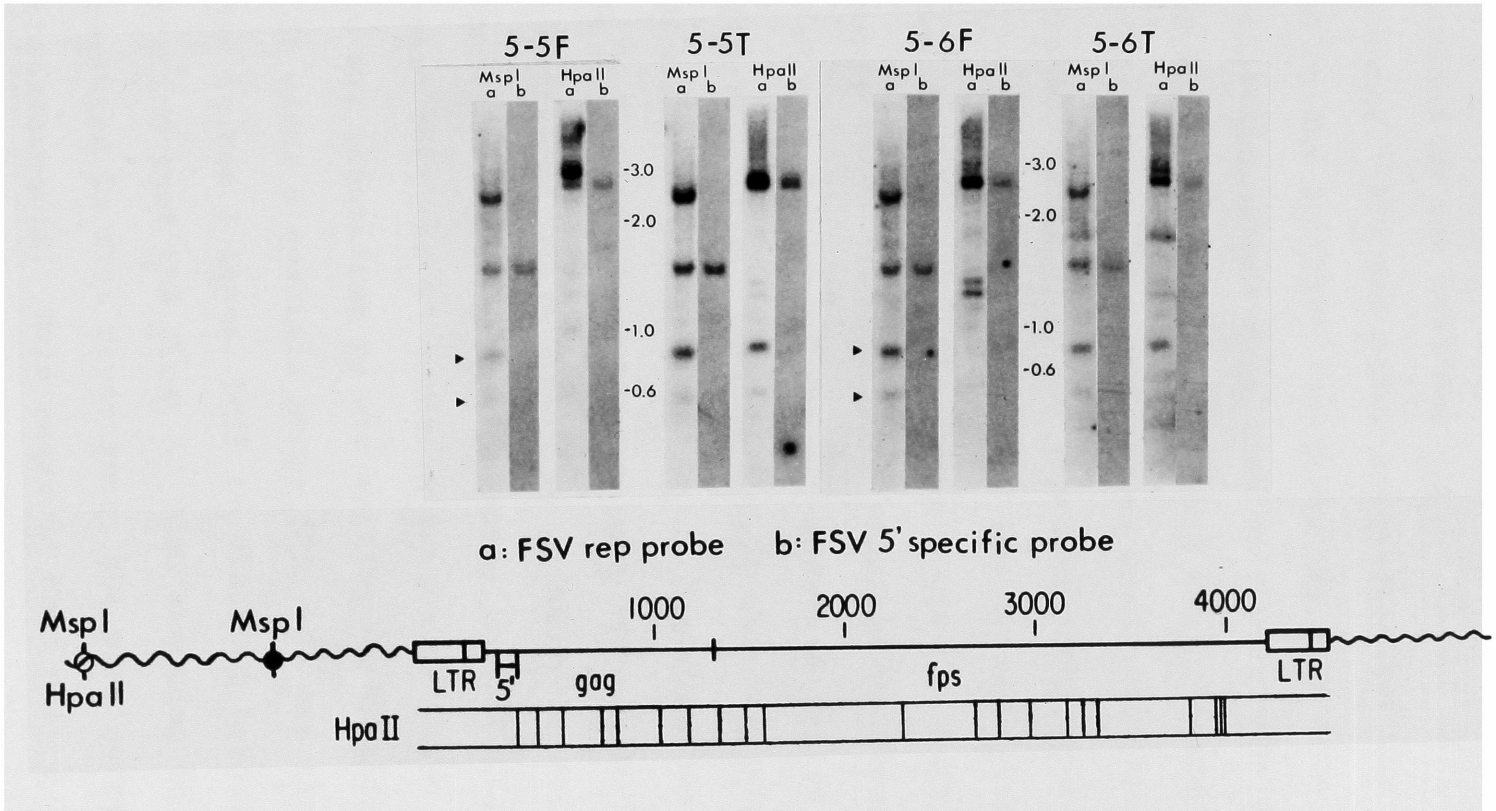


Figure 7. Hybridization of $cDNA_{fps}$ to cellular RNA in class III revertants and their retransformants.
Total cellular RNA was extracted from uninfected cells, and the indicated revertants and retransformants. Various amount (0.32 ug to 100 ug) of cellular RNA were incubated with 500 cpm of ^3H-labeled $cDNA_{fps}$ in 8 to 16 ul of hybridization solution. The reaction was carried out for 100 h under conditions of moderate stringency (50 C, 30% formamide, 0.45 M NaCl, 45 mM sodium citrate, 5 mM EDTA and 0.1% SDS). Extent of hybridization was measured by S1 nuclease digestion. x: 3Y1 cells; ▽ :5-3F; △ 5-4F; □:5-5F; ○:5-6F; ▼:5-3T; ▲:5-4T; ■:5-5T; ●:5-6T.

As shown in Fig. 7, no fps mRNA was detected in uninfected 3Y1 cells, as well as in three revertants (5-3F, -4F, -5F), even at the highest C_rt value. Revertant 5-6F, which showed also detectable amount of P130, expressed about 3 to 5 copies of viral RNA per cell. This contrasted sharply with the levels found for the retransformants, which ranged from 60 to 100 copies of RNA per cell. Thus, reversion in these clones resulted from a transcriptional block of the FSV provirus, which defined what we called class III revertants.

Methylation of FSV proviral DNA: There exists an inverse correlation between DNA methylation and gene expression (reviewed in [100]). DNA of actively transcribed genes is usually hypomethylated, whereas untranscribed genes have their DNA hypermethylated. To check whether this correlation was also observed in the case of class III revertants and their retransformants, we digested total DNA isolated from these clones with the isoschizomers Hpa II and Msp I, which recognize the same sequence CCGG. However, when the internal C of this sequence is methylated, only Msp I will be able to cut [135]. Fig. 8 shows the results obtained with two pairs of revertants and their retransformants, 5-5F, 5-5T and 5-6F, 5-6T. The nucleotide sequence of the FSV provirus [117] predicts that digestion of proviral DNA with Msp I should generate four identifiable fragments.

Figure 8. DNA methylation of FSV proviral DNA in class III revertants and their retransformants.
High molecular weight DNA of clones 5-5F, -6F and 5-5T, -6T was digested with Msp I or Hpa II (as indicated above each lane), separated on a 1.5% agarose gel, transferred to nitrocellulose paper and hybridized to FSV_{rep} probe (lanes a); the hybridized probe was removed by washing the filter at 65 C in low salt buffer and the blot was rehybridized to the Sau 3A-Hpa II 5'-specific probe (labeled 5' in diagram) (lanes b). Schematic diagram of FSV provirus (solid line flanked by open boxes). Hpa II (or Msp I) sites are indicated by vertical bars. The Msp I and Hpa II sites indicated in the 5' flanking, cellular sequence are not drawn to scale. (o: unmethylated site; ●: methylated site.)

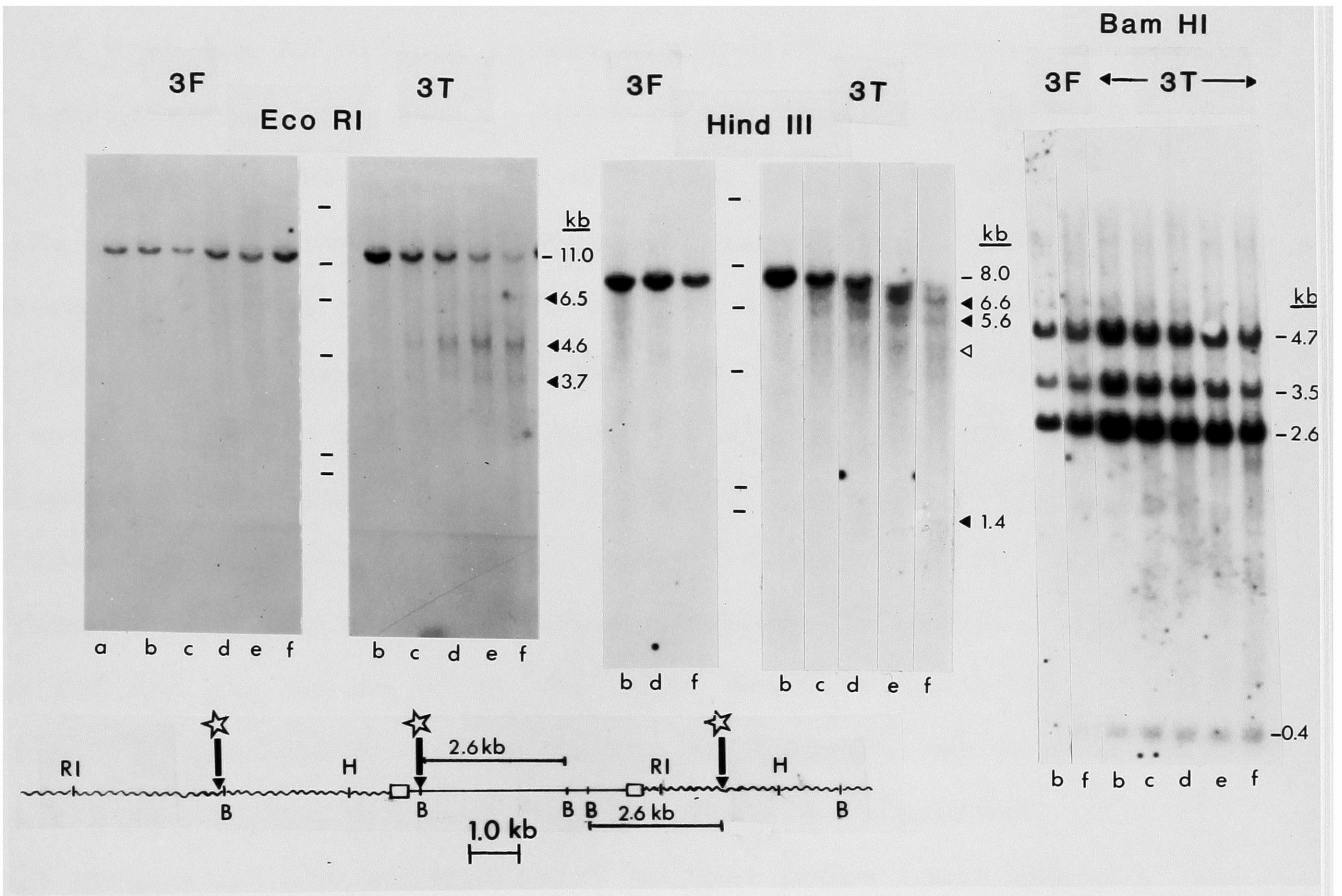

5-5F
5-5T
5-6F
5-6T
Msp I
Hpa II
a
b
-3.0
-2.0
-1.0
-0.6
a: FSV rep probe
b: FSV 5' specific probe
Msp I
Msp I
Hpa II
1000
2000
3000
4000
LTR
5'
gag
fps
LTR
Hpa II

These are an 0.7 and an 0.4 kb fragment derived from fps sequence, and a 5' and 3' junction fragment of a length which will be determined by the next Msp I site in the cellular flanking sequences. All the other fragments were too small to be identified by the Southern analysis. We were able to confirm that prediction, since four fragments were seen for both revertant and retransformant DNA, when a FSV_{rep} probe was used for the hybridization. When Hpa II was used, only the DNAs from the retransformants gave the expected internal 0.7 and 0.4 kb fragments, results consistent with the hypomethylation of their proviral DNAs. DNAs of revertants yielded high molecular weight fragments which were not well resolved, indicating a high degree of methylation of the FSV provirus and of the cellular flanking sequences. Revertant 5-6F which expressed small but detectable amount of viral RNA, was characterized by DNA fragments of intermediate size which were absent in clone 5-5F. This lesser degree of DNA methylation probably correlates with the weak expression of viral RNA observed in this clone. It was of interest that the 5' junction fragment, shown in the b lanes, which was identified by its ability to hybridize to a Sau 3A-Hpa II viral 5'specific probe (Fig. 8) was larger in Hpa II lanes than in Msp I lanes, both in flat and transformed cells, indicating that the first 5' CCGG sequence in flanking DNA was methylated in flat revertants, but more surprisingly also in retransformants. This suggests that only a small region limited to the proviral DNA may be hypomethylated in transformed cells.

The frequency of spontaneous retransformation is rather low in these clones (10^{-6}-10^{-7}). To see whether we could increase this frequency, we treated the clones with 5-azacytidine, a cytosine analog which cannot be methylated at the 5 position. We observed some increase in the frequency of retransformation (Table 1), suggesting that retransformation was linked to demethylation of FSV proviral DNA.

Chromatin structure of FSV proviral DNA: Actively transcribed genes have been shown to be more susceptible to DNase I digestion and to possess DNase I hypersensitive sites, whereas untranscribed genes are relatively resistant even to high concentration of DNase I and they do not contain hypersensitive sites [140,141]. To test whether the relationship between DNase I sensitivity and gene activity was also applicable to FSV provirus, we isolated nuclei from the revertant clone 5-3F and its retransformant 5-3T, and treated them with increasing concentration of DNase I. DNA was isolated and digested with Eco RI or Hind III which do not cut the FSV provirus, and with Bam Hl which cuts three times in the provirus. The digested DNAs were subjected to a Southern analysis using a FSV_{rep} probe. The Eco RI panel in Fig. 9 shows that the FSV provirus of the revertant clone 5-3F is quite resistant to DNase I digestion. There were no specific subbands that were evident even at the highest DNase I concentration. In the retransformant 5-3T, the provirus was susceptible to DNase I digestion and three subbands of increasing intensity with increasing DNase I concentration were detected.

Figure 9. DNase I hypersensitivity of proviral DNA in clones 5-3F and 5-3T.
Nuclei from clones 5-3F and 5-3T were treated with increasing amounts of DNase I. DNA was extracted and cut with various enzymes as indicated in the figure. DNA digests were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to FSV_{rep} probe. Lane a: 5-3F control DNA; DNase I concentrations: 0 ug/ml (b); 0.1 ug/ml (c); 0.2 ug/ml (d); 0.4 ug/ml (e) and 0.8 ug/ml (f). Solid arrowheads: DNase I specific subbands; open arrowhead: DNase I non specific subband. Size markers are included in Eco RI and Hind III panels (λ cut with Hind III: 23.3 kb; 9.5 kb; 6.4 kb; 4.2 kb; 2.2 kb; 1.8 kb) Schematic diagram of FSV provirus (solid bar flanked by open boxes) and cellular flanking sequences (wavy line). RI: Eco RI sites; B: Bam HI site; H: Hind III sites. DNase I hypersensitive sites in clone 5-3T are indicated by a star and arrow. The 2.6 kb comigrating fragments (the internal Bam HI FSV fragment and the Bam HI-DNase I (3' site) junction fragment) are indicated.


Bam HI
3F
3T
Eco RI
3F
3T
Hind III
3F ← 3T →
kb
11.0
6.5
4.6
3.7
a b c d e f
b c d e f
kb
8.0
6.6
5.6
1.4
b d f
b c d e f
kb
4.7
3.5
2.6
0.4
b f b c d e f
RI
H
B
2.6kb
1.0 kb

Results with Hind III confirmed the presence of hypersensitive sites in the provirus of 5-3T, and in this case two subbands were observed. The fuzzy subband, denoted by an open arrowhead (Fig. 9, Hind III panel) seemed to be present both in 5-3F and 5-3T proviral DNAs, and may represent c-fes (which we can detect easily if we use a fps-specific probe). Digestion of total DNA with Bam Hl did not reveal any specific subband, after hybridization with FSV_{rep}, even in the case of clone 5-3T (Fig. 9). In order to understand this last result, we undertook the mapping of the DNase I hypersensitive sites. First, we determined the Eco RI, Hind III and Bam Hl sites in the cellular flanking regions of the FSV provirus. Total DNA was digested with Eco RI and Bam Hl, or Hind III and Bam Hl, and the DNA fragments were hybridized to a 5' or 3' viral-specific probe (see Material and Methods). Second, we rehybridized the blot shown in the Eco RI panel with a gag-specific probe, which recognized the gag region 3' to the first Bam Hl site in the provirus. In addition to the 11 kb Eco RI fragment, we detected the 4.6 kb subband and very faintly, the 6.5 kb subband. The 3.7 kb subband did not hybridize to that probe (data not shown). Third, the blot obtained after Bam Hl digestion was rehybridized to a 3' viral-specific probe. In this case, we detected the expected 4.7 kb junction fragment, but also a faint subband at 2.6 kb (data not shown). This subband was only seen at higher concentration of DNase I in 5-3T proviral DNA. By combining these results and the size of the different subbands observed after Eco RI and Hind III digestion, we were

able to determine the location of three DNase I hypersensitive sites in the chromatin region containing the FSV provirus of retransformant 5-3T (see diagram, Fig. 9). One DNase I site coincides with a Bam H1 in proviral DNA (within a few nucleotides). The 3' DNase I site generates a Bam-DNase I subband which exactly comigrates with the internal Bam-Bam 2.6 kb fragment. Finally, the 5' DNase I site lies just upstream of the Bam H1 site, present in the 5' flanking sequence, and therefore this Bam H1 junction fragment would not be shortened by DNase I digestion. In revertant clone 5-3F, we did not detect any DNase I hypersensitive site within a region covering 14 kb, and which contained the FSV provirus.

Superinfection of revertants: Revertants of the three classes were susceptible to retransformation by FSV (Table 1). To avoid any ambiguity in the analysis of superinfected clones, we used the strain of FSV coding for P140. In the case of class III revertants, superinfection did not result in the activation of the resident provirus (which is transcriptionally inactive in these revertants), since only P140 could be detected in these cells (data not shown). Revertant clones and uninfected 3Y1 cells could be transformed by FSV with similar efficiency, and equal amount of P140 was found in these cells (data not shown).

Table 1. PROPERTIES OF 3Y1-FSV CLONES

	Original Transformants	Partial Revertants	Flat Revertants			Retransformants
		Class III	Class I	Class II	Class III	
	3	3/3	3/15	1/15	11/15	11/11
Provirus/Cell	1 copy	1	0	1	1	1
[a]fps mRNA/Cell	60-100	10-30	0	60-100	< 3	60-100
P130/Cell	+++	+	-	+++	-	+++
Kinase Activity	+++	+	-	-	-	+++
DNA Methylation					High	Low
[b]Retransformation by 5-Azacytidine			No	No	Yes	
[c]Superinfection with FSV			Yes	Yes	Yes	

[a]Copies of fps RNA were calculated as described in Material and Methods. $C_rt_{1/2}$ values were obtained from the data presented in Figure 7.

[b]Cells were treated with 5 uM of 5-azacytidine for 48 h; the medium was changed, and foci were counted after 3 weeks.

[c]The FSV strain used for superinfection coded for P140.

Intermediate transformants: In the process of screening revertants, we isolated three partial revertants which showed intermediate morphological transformation and expressed intermediate levels of viral RNA and P130 protein. To see whether different degree of transformation could be correlated with different levels of viral RNA, we decided to extend this observation to a larger number of clones. We infected 3Y1 cells with FSV and selected foci which differed in their extent of morphological transformation. These foci were cloned and grown to mass culture, examples of which can be seen on Fig. 10. These cultures displayed different degrees of morphological transformation, ranging from an almost normal-like appearance to a frankly transformed one. We quantitated by liquid hybridization the amount of viral RNA expressed in these cultures. As shown in Table 2, the copy number of v-fps mRNA varied from 10 to 80 copies per cell, and clearly the level of the viral RNA correlated with the degree of morphological transformation. Therefore, transformation by FSV can be said to be the result of the high expression of its viral product P130, but more importantly a low level of P130 is not sufficient to commit the cells to a transformed state.

Figure 10. Variations in morphology of FSV-infected clones. 3Y1 cells were infected with FSV. Foci showing variable extent of phenotypic transformation were selected, and cells derived from each independent focus were cloned. Electromicrographs of uninfected rat fibroblasts (3Y1) and of individual FSV-infected clones derived from foci #22, #23, #29, #31, #33.

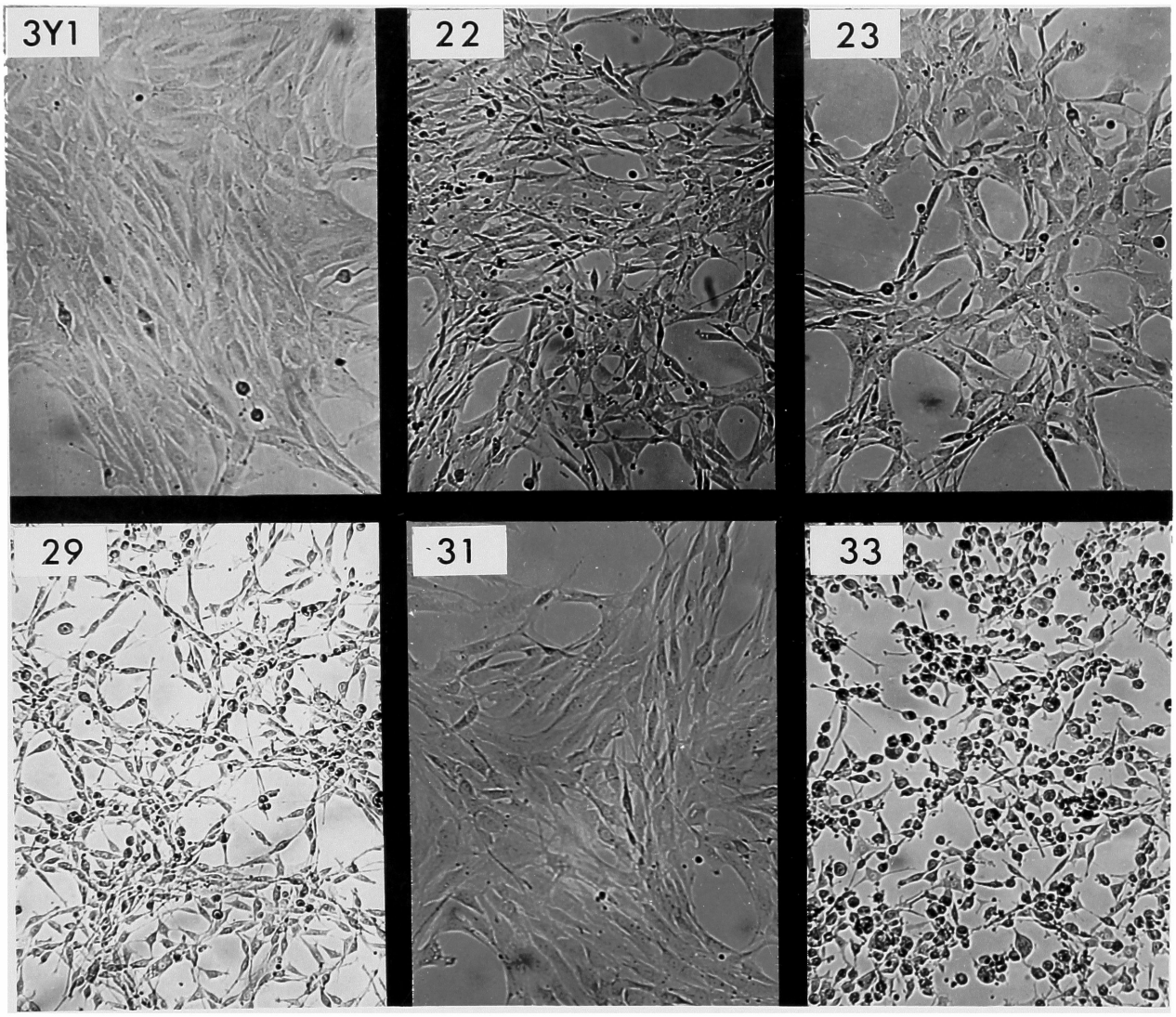

3Y1
22
23
29
31
33

Table 2. v-fps mRNA in FSV-infected rat cells showing various extents of phenotypic transformation

Cells[a]	Transformed phenotype	RNA content (copies/cell)[b]
3Y1	-	0
5G	+/-	7
5H	+	19
21A	+/-	11
22J	+	17
22G	++	28
23J	+/-	10
23I	++	15
25A	+	51
26G	+++	80
26B	++++	125
28A	+/-	23
29B	++	46
29E	++++	80
30E	+/++	54
31B	+	20

Total cellular RNA was extracted from uninfected cells (3Y1), or FSV-infected clones of rat fibroblasts (5G,5H,21A,22G, 22J,23I,23J,25A,26B,26G,28A,29E,30E,31B).

[a]The nomenclature of the clones is as described in the legend of Figure 1.

[b]Calculation of copies of RNA per cell is as described in Material and Methods. The conditions of hybridization are as described in the legend of Figure 7.

DISCUSSION

Table 1 summarizes the data we obtained for the revertants. Fifteen independent revertants were isolated from three original foci. They fell into three different classes. Class I was characterized by the loss of provirus (3 clones). Class II had a mutated provirus which encoded a transformation defective protein, P130 td (1 clone). Class III showed a transcriptional block of the provirus, which was reversible spontaneously at low frequency (11 clones). All revertants could be retransformed after superinfection with FSV (Table 1), ruling out the possibility that reversion was the result of a cellular mutation. Due to the low probability of having a mutation affecting simultaneously a pair of cellular alleles which are involved in some stage of the oncogenic process, these cellular revertants have been extremely rare. To date, only two examples of this type of mutants have been reported in retrovirus-infected cells [67, Bassin, personal communication], and in one case, reversion to the normal phenotype was in fact the result of a reduced kinase activity of $p60^{src}$ combined with the inability of the cells to be transformed or tumorigenic [67].

The types of flat revertants we obtained in this study are not unique to FSV, since the same classes have been described in cells infected with feline sarcoma virus [4,30,97] and with Rous sarcoma virus or B77 [8,16,29,88,90,132].

Excision of provirus: although class I is, conceptually, a simple case of reversion, very little is known, mechanistically,

about how the loss of the provirus occurs. Many of the properties of retroviruses have prompted some speculation about structural similarities between proviruses and transposable elements (see [129] for review). Since proviruses have redundant ends, carrying inverted and short direct repeats, the loss of the provirus might result from its precise excision, by recombination between the terminal repeats. However, Varmus et al. reported that partial deletion of the retroviral genome can take place in some revertants [132], indicating that the loss of viral sequences need not result from recombination between LTRs. In our case, we observed the deletion of the complete copy of the provirus; whether some cellular flanking sequences, or even the whole chromosome have been also lost, still remains to be determined.

Provirus mutation: clone 12-2F expressed a transformation-defective protein, P130 , which lacked protein kinase activity in vitro. Incubation of cells at 32 C had no effect on their morphology, and did not result in the reactivation of the enzymatic activity normally associated with the transforming protein of FSV (data not shown). An interesting aspect of 12-2F P130 is its association with the two cellular proteins, 90K and 50K. These two proteins can be found in association in vivo with the transforming proteins of avian sarcoma viruses, and this binding is greatly enhanced in ts mutant-infected cells [11,12,22], but the role of this complex remains unclear. Recently, Brugge and others have shown that newly synthesized $p60^{src}$ was predominantly found in the complex, and suggested

that it might mediate the transport of $p60^{src}$ to the cell membrane [12,23]. In addition, they found that $p60^{src}$, in this complex, had very little kinase activity. Moreover, in ts mutant-infected cells, most of $p60^{src}$ was found to be in the cytoplasmic fraction, in association with 90K and 50K [12,23]. In this respect, it is important to determine whether the flat phenotype observed in clone 12-2F reflects intrinsic properties of the mutant P130 (i.e. no kinase activity) or whether this characteristic of 12-2F is a consequence of the stable binding of P130 to 90K and 50K. In the superinfected clone 12-2ST1, P130 was phosphorylated on tyrosine residues, both in vivo and in vitro, suggesting that it is a substrate of P140. However, when a cell extract of 12-2F was mixed, prior to immunoprecipitation, with an extract of FSV-infected 3Y1 cells expressing P140, only P140 was labeled in the in vitro kinase reaction. If the lack of protein kinase activity in P130 were due to the binding of 90K and 50K, it possible that the phosphorylation observed for P130 in clone 12-2ST1 reflects the presence of "free" P130, which could undergo autophosphorylation. However, by comparing the intensity of ^{35}S-labeled 90K and 50K found in immune precipitates of extracts of clones 12-2F and 12-2ST1, we found the same amount of these proteins relative to 12-2F P130. Therefore, if the in vitro phosphorylation of 12-2F P130 were due to the presence of the small amount of free P130 (which could undergo autophosphorylation), we should expect a marked difference in the relative intensity of 12-2F P130 and P140 labeled in vitro. Fig. 4 shows that it is not the case, since

the two proteins seem to be equally phosphorylated. In any case, we cannot fully explain the discrepancy of these results and experiments are under way to solve this problem.

Although the size of 12-2F P130 is nearly identical to that of wild-type P130 , we believe that the 12-2F provirus has suffered some small deletion, since even after three months of constant passings, no spontaneous retransformant has been observed, making a point mutation, as the defect in 12-2F provirus, very unlikely. We are attempting to clone the FSV provirus of 12-2F, in order to localize this mutation.

Alteration in transcription: an inverse correlation between gene activity and methylation has been decribed in several systems (see [100,141] for review), including retroviruses [17,31,40,46,55,123]. This correlation, however, has not been absolute [81], and it seems as if the presence of certain methylated domains, especially in the 5' region of a gene, might correlate better than the overall extent of methylation [74,79,106]. In this respect, we found that the retransformants, even though they had retained a 5' junction fragment hypermethylated (Fig. 8), expressed a high amount of v-fps mRNA. In this case, hypomethylation seemed to be restricted only to the proviral DNA. However, in the clones derived from focus #5, the first CCGG site is about 1 kb upstream of the 5' LTR; therefore, we cannot rule out the possibilty that the key region for regulation of transcription by methylation lies between this site and the LTR. Finally, methylation of DNA must affect only localized regions of the host genome, since

superinfection with FSV , caused retransformation of these cells, with the same efficiency as with uninfected 3Y1 cells and without the concomitant reexpression of P130, the product of the resident provirus.

Like cellular genes, the expression of retroviral genomes can be correlated with the conformation of these sequences in chromatin [17,46,123]. In retransformants derived from class III revertants of cells, originally transformed with B77, Chiswell et al. described the presence of two new hypersensitive sites in the B77 provirus of the retransformants [17]. In particular, one site was mapped in the LTR, confirming an observation made by Groudine et al., who located the hypersensitive sites of the transcriptionally active ev-3 avian endogenous retrovirus in the LTRs [46]. We detected three sites located within and around proviral DNA in the retransformant, but not in the LTRs of FSV. Two sites lay in the flanking sequences, whereas one was located in the gag region (around nucleotide 535, [117]) of the provirus. We did not detect any hypersensitive sites in the revertant, and since the retransformant was derived from the former, hypersensitive sites were generated de novo. It would be interesting to see, whether the same hypersensitive sites were present in the original focus #5.

In partial retransformants, we observed that weak to moderate expression of viral fps mRNA correlated with low to intermediate states of morphological transformation. This argues for the model [97] in which FSV transformation is a function of the dosage of the viral RNA, and does not consist in an

"all or none" phenomenon. Furthermore, the strong promotor activity, which is associated with sequences present in the LTR and regulates the transcription of FSV, can be influenced or even superseded by cellular signals. DNA methylation and chromatin structure may constitute such signals, or be manifestations of them. How exactly these signals influence or relate to transcriptional activity, and to what regulation they themselves obey, remain to be determined.

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End