Studies on the Structure and Function of the Avian Sarcoma Virus Transforming Gene Product

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A. Introduction

RNA tumor viruses quickly and efficiently transform cells and are therefore extremely useful agents for the study of the molecular events in oncogenesis. It is well established that the product(s) of a single avian sarcoma virus (ASV) gene *(src)* is responsible for the induction and maintenance of cell transformation in vitro and tumor production in infected animals [6]. We have carried out experiments designed to identify this product by techniques that require no assumptions concerning its mechanism of action. This approach was necessary because we anticipated that the *src* protein would be present at relatively low levels in the transformed cells, therefore making direct identification impossible. For example, the precursor to the major virion structural proteins is present at such low levels it can be identified only by immunoprecipitation [5]. Furthermore, since there are such a variety of biochemical changes reported to occur in transformed cells, it is difficult to predict at what level the *src* gene product might disrupt normal cellular processes. This, in turn, makes an accurate forecast concerning its function unlikely.

Recent work in our laboratory has resulted in the identification of a phosphoprotein with a molecular weight of 60000 that appears to be the product of the ASV *src* gene. A summary of that evidence is presented in Table 1. Because most of the experimental results that support these statements have been published [2, 3, 12, 13], they will not be reviewed here. We feel that it is consistent with our results to designate the 60000 molecular weight phosphoprotein, $p60^{src}$, the product of the *src* gene.

Table 1. Evidence that p60 is the product of the avian sarcoma virus transforming gene

I. A transformation-specific phosphoprotein with a molecular weight of 60000 is found in ASV-transformed cells by immunoprecipitation with antiserum from tumor-bearing rabbits.

II. The polypeptide p60 is not found in cells infected with ASV mutants which have a deletion in the transforming gene.

III. Immunoprecipitation of p60 does not depend on antibody directed against virion proteins.

IV. The p60 polypeptides found in all avian and mammalian cells transformed by the Schmidt-Ruppin strain of ASV are biochemically similar.

V. In vitro translation of viral RNA which contains the transforming gene produces a p60 immunologically and biochemically similar to that found in transformed cells.

The major tools employed in these studies were a) cell-free translation of that region of the viral RNA which contains the *src* gene and b) immunoprecipitation carried out with antiserum from rabbits bearing ASV-induced fibrosarcomas (TBR serum). The latter approach is one that has proved useful in the identification of non-structural proteins encoded by DNA-containing tumor viruses. The availability of antiserum that recognizes the product of the ASV *src* gene provided an essential reagent for the design of experiments for its purification and the characterization of its function. We will describe here recent results concerning the function of the ASV *src* gene product.

B. Results

I. Preliminary Observations

We were able to prepare antiserum that was monospecific for $p60^{src}$ and carry out immunofluorescence studies and found that transformed cells generally exhibited a diffuse cytoplasmic fluorescence without a strong association with any specific structures (Paula Steinbaugh and Joan S. Brugge, unpublished data). The nucleus of transformed cells was negative as was the cytoplasm of uninfected cells or cells infected with ASV mutants which have *src* gene deletions. Consistent with these observations were studies which showed that $p60^{src}$ appeared in cytoplasmic but not nuclear fractions when transformed cells were fractionated with detergents prior to immunoprecipitation. Additional information showed that $p60^{src}$ was readily solubilized in the absence of detergent by sonication of transformed cells. Taken together, these observations suggested to us that $p60^{src}$ might be an enzyme localized in the cytoplasm. In considering enzymes known to play a role in regulation of cellular functions, protein kinases were ready candidates. We could speculate, for example, that *src* encoded a protein kinase, which when expressed at relatively high levels produced a cellular alteration(s) that results in the transformed phenotype.

II. Correlation of the Immunoprecipitation of $p60^{src}$ and Protein Kinase Activity

To determine if immunoprecipitates containing $p60^{src}$ exhibited protein phosphorylating activity, immune complexes (formed with the protein A-bearing bacterium *Staphylococcus aureus* [7]) were resuspended directly in a protein kinase reaction mixture [4]. These complexes were incubated with [γ-^{32}P] ATP in the presence or absence of exogenous substrates. The reaction was terminated by adding SDS-containing buffer, the bacteria were separated from the IgG and antigens, and the latter subjected to SDS/polyacrylamide gel electrophoresis. Under these assay conditions exogenous substrates, such as histone and casein were not phosphorylated, but IgG was phosphorylated. The phosphorylated IgG was found to contain a single tryptic phosphopeptide and exclusively phosphothreonine.

Table 2. Correlation of the protein kinase activity and the immunoprecipitation of p60^{src}

Virus	Cell	Trans-formation	p60^{src}	Protein kinase activity
—	Chick	–	–	–
RAV-2	Chick	–	–	–
td-SR-ASV	Chick	–	–	–
SR-ASV	Chick	+	+	+
SR-ASV	Hamster	+	+	+
SR-ASV	Hamster revertant	–	–	–
—	Vole	–	–	–
SR-ASV	Vole	+	+	+
Pr-ASV	Chick	+	–	–
B77-ASV	Chick	+	–	–
BH-ASV (RAV-50)	Chick	+	–	–

Uninfected cell cultures or cultures infected with various strains of ASV were either radiolabeled with [^{35}S] methionine (for detection of p60^{src}) or left unlabeled (for detection of protein kinase activity). Cells were lysed and each extract (1800–3600 μg of protein) was immunoprecipitated with TBR serum and a portion of the bacteria-bound immune complexes was incubated in the protein kinase reaction mixture.
The reaction mixtures (25 μl) contained 20 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, and 0.4–1.2 μM [γ-^{32}P] ATP (1000 Ci/mMol). After termination of the reaction by heating to 95° for 1 min. in SDS-containing buffer and pelleting of the bacteria, the supernatant was subjected to electrophoresis in a discontinuous SDS/polyacrylamide slab gel [9]. Autoradiography was used to detect the presence (+) or absence (–) of ^{35}S-methionine-labeled p60^{src} and phosphorylated IgG.

The immunoprecipitation of p60^{src} by the rabbit antiserum used in these experiments is strain-specific. Antibody produced in rabbits bearing tumors produced by Schmidt-Ruppin (SR) ASV does not immunoprecipitate a transformation-specific polypeptide from chick cells transformed by other strains of ASV. This fact permitted us to test the specificity of, and association of, the protein phosphorylating activity with the presence of p60^{src} in a variety of circumstances. The results of these experiments are summarized in Table 2. These results show that protein kinase activity is immunoprecipitated from cell extracts only when p60^{src} is also immunoprecipitated. The activity is not found in untransformed cells or in cells infected with strains of ASV which lack a *src* gene and which therefore do not cause cellular transformation. Furthermore, no activity is found in immunoprecipitates of extracts of transformed cells when we are unable to immunoprecipitate p60^{src} because of the specificity of the TBR serum. However, when antiserum from tumor-bearing marmosets [10], which crossreacts with the *src* gene product encoded by other strains of ASV (unpublished data), is used in this assay, protein phosphorylation is found in the precipitates (Table 3). No enzymatic activity is ever observed in complexes formed with normal serum [4].

A further indication that the protein kinase activity is the result of expression of the ASV *src* gene is shown by the observation that the expression of the phosphorylation activity observed is thermosensitive in chick cells infected with a temperature-sensitive *(ts)* mutant in the *src* gene. Parallel cultures of chick cells infected with nondefective (nd) SR-ASV and the SR-ASV

Table 3. Immunoprecipitation of p60^{src} and protein kinase activity from transformed chick cells by serum from tumor-bearing marmosets

Virus	p60^{src}	Protein kinase activity
SR	+	+
PrC	+	+
B77	+	+
Bryan	+	+

ts mutant NY68 [6] were transformed and then grown for 16 hours at either 35° or 41°. Cell extracts were prepared from each of the four cell cultures, immunoprecipitated with TBR serum, and then analyzed for protein kinase activity. The results show (Table 4) that cells infected with nondefective ASV yielded slightly more (2-fold) phosphorylating activity when grown at 41° compared to those grown at 35°. This is in sharp contrast to NY68-infected cells which show a dramatic decrease in phosphorylation activity when grown at the nonpermissive temperature, 41°.

Table 4. Growth temperature-dependent expression of src protein-immunoprecipitated phosphorylating activity in chick cells infected with a ts transformation mutant of ASV

Virus	Growth temperature, °C	*Phosphorylating activity* ^{32}P incorporated, fmol/mg protein	Normalized values
SR-ASV (nd)	35	16.6	1.00
SR-ASV (nd)	41	35.8	2.16
SR-NY68	35	19.8	1.00
SR-NY68	41	1.7	0.09

Parallel cultures of chick cells infected with nd SR-ASV and cells infected with the ts transformation mutant of SR-ASV, NY68, were maintained at either the permissive (35°) or nonpermissive (41°) temperature. Cell extracts were prepared from the four cultures, samples were taken for determination of protein content, and the remainder was immunoprecipitated with TBR serum as described in Table 2. Phosphorylating activity by the bacteria-bound immunoprecipitated complexes was also determined. The resulting activity values, determined by quantitation of the phosphorylated IgG bands from a polyacrylamide gel, are normalized with respect to the amount of cell extract protein used for immunoprecipitation and the activity present in the respective cells grown at 35°.

III. In Vitro Synthesis of p60^{src} that Functions in the Phosphotransferase Reaction

Radiolabeled p60^{src} has been synthesized in cell-free extracts programmed by the 3′ third of viral RNA, the region of the genome which contains the *src* gene, but not by similar RNA from a mutant which has a deletion in the *src* gene [12, 13]. Because the in vitro and in vivo products of the *src* gene were structurally similar [13] we also tested the in vitro translation product for phosphotransferase activity. The 70S RNA from nondefective and from

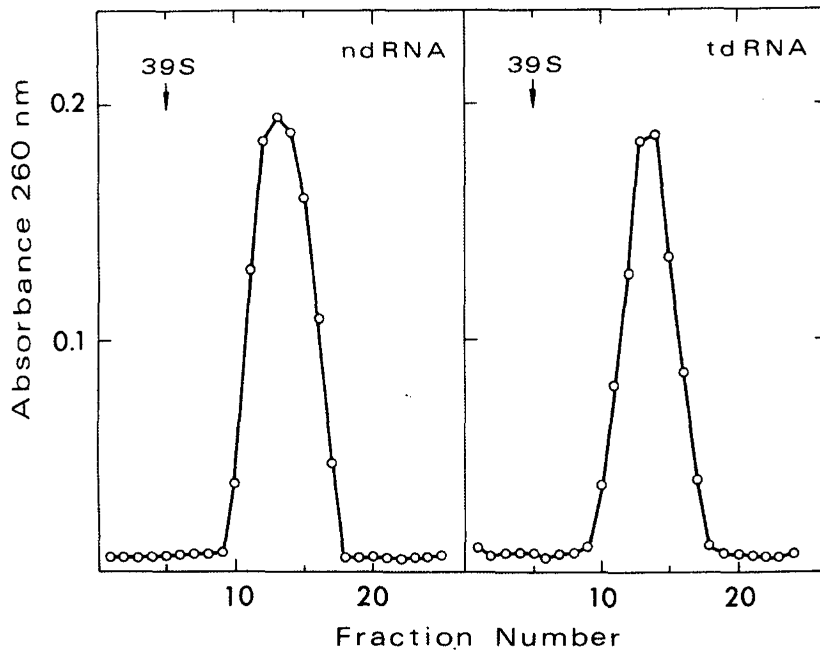

Fig. 1. Sedimentation profile of nd and td poly A-containing virion RNA selected fro translation. RNA selected from a previous sucrose gradient was precipitated with ethanol, dissolved in EDTA 1 mM, Tris-HCl 0.01 M, pH 7.2, heated at 80°C for 2 min, quick chilled and sedimented for 105 min through a 20 to 5% (W/V) sucrose gradient containing 0.1 M NaCl at 10°C and 45 000 rpm in a Beckman SW 50.1 rotor. The RNA in fraction numbers 12–15 was ethanol-precipitated and used for cell-free translation as described in the legend to Fig. 2

transformation-defective (td) virions was purified, heat denaturated and the poly A-containing RNA was purified by oligo-dT cellulose chromatography. The 3′ third of the genome was selected from this RNA by sucrose gradient sedimentation. This RNA was resedimented (Fig. 1) and the RNA in fraction numbers 12–15 was used to program messenger RNA-dependent reticulocyte lysates as described in the legend to Fig. 2. The ^{35}S-methionine-labeled polypeptide translation products analyzed directly by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis show that $p60^{src}$ is translated only from nd RNA (Fig. 2, tracks 1–3). No $p60^{src}$ is translated from td RNA although this RNA stimulated protein synthesis as well as did the nd RNA, both about 10-fold above background. We have not studied the nature of the polypeptides synthesized in response to td RNA, except that one migrates with Pr76, the product of the ASV *gag* gene.

TBR serum was used to immunoprecipitate the in vitro translation products by the same procedures used to precipitate $p60^{src}$-associated protein kinase from transformed cell extracts (Table 1 and reference [4]). The radiolabeled polypeptides found in immunoprecipitates are displayed in Fig. 2, tracks 4–7. TBR serum precipitates $p60^{src}$ and a small amount of Pr76 from the products synthesized in response to nd RNA (track 5). A small amount of Pr76 is found in the immunoprecipitate of the products synthesized in response to nd RNA (track 5) and to td RNA (track 7). This indicates that radiolabeled virus-specific polypeptides synthesized in cell-free extracts are antigenically similar to those found in transformed cells [2,3].

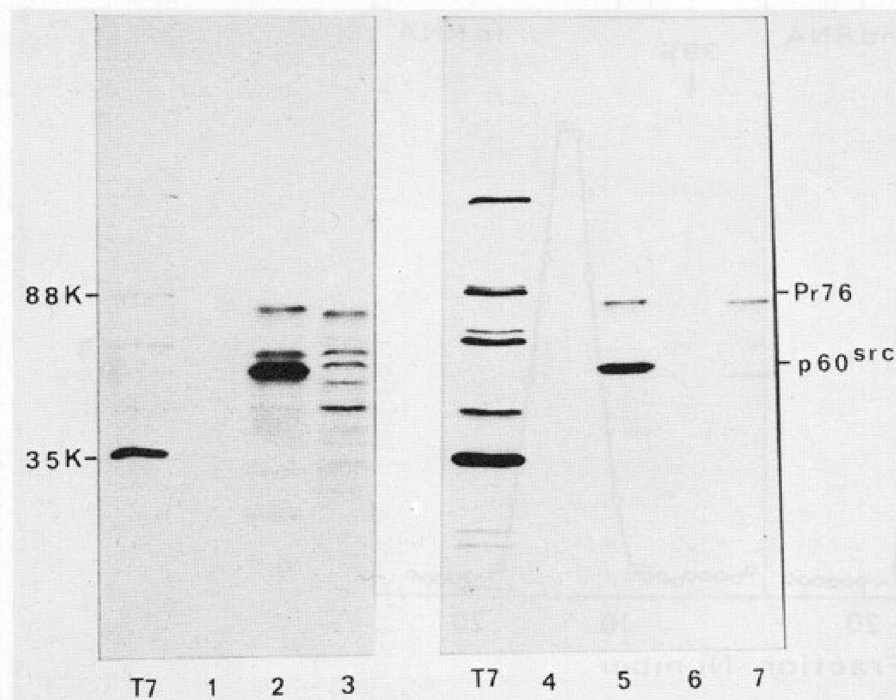

Fig. 2. Fluorogram of SDS-polyacrylamide gel electrophoresis analysis of ^{35}S-methionine-labeled polypeptides synthesized in cell-free extracts programmed by the RNA shown in Fig. 1. Messenger-dependent reticulocyte lysates were prepared as described by Pelham and Jackson [11] and the conditions of translation were as described [13] except that the final concentrations were 50mM KCl. 10mM Tris-HCl (pH7.4), 1mM Mg acetate, 100 μM unlabeled methionine, and 0.5 μCiμl^{-1} ^{35}S-methionine (700 Ci mM^{-1}. New England Nuclear). Three picomoles of nd RNA was added to each of two 300-μl reaction-mixtures, and 3 picomoles of td RNA was added to one 300 μl reaction-mixture, and the reaction-mixtures were incubated at 30°C for 40 min. At this time samples were taken for the determination of incorporation, for polyacrylamide gel analysis, and one-half of each reaction was used for determination of enzymatic activity. Stimulation of 0.3 N KOH (15 min at 37°C)-resistant and 10% TCA-precipitable radioactivity was 10-fold above background for the lysate programmed with nd RNA and 9-fold for tha programmed with td RNA. The direct analysis of these translation products is shown in tracks 1. no RNA, 2. nd RNA and 3. td RNA. Immunoprecipitation of the translation products was carried out with normal rabbit serum or TBR serum and the immunoprecipiated polypeptides are shown in tracks 4. nd RNA, normal serum, 5. nd RNA, TBR serum, 6. td RNA, normal serum, and 7. td RNA, TBR serum. Immunoprecipitations were carried out as previously described [2] using the procedure of Kessler [7]. Prior to electrophoresis, samples were boiled in sample buffer (0.07 M Tris-HCl, pH6.8, 11.2% glycerol, 3% SDS, 0.002% bromophenol blue, 5% β-mercaptoethanol). T_7 virion proteins are included as molecular weight markers

A portion of the immune complexes formed with the translation products shown in Fig. 2 was also resuspended directly in kinase reaction buffer, and the transfer of phosphate from γ-labeled ATP to the heavy chain of IgG was analyzed by SDS-polyacrylamide gel electrophoresis of the reaction products. As seen in Fig. 3, only the products of translation of nd RNA immunoprecipitated with TBR serum yielded phosphorylated IgG (Fig. 3, track 4). Translation of the same amount of td RNA resulted in no detectable enzymatic activity with immune serum (Fig. 3, track 3). These results, as well as those given in Tables 2 and 3 and those previously published, indicate directly that only immune complexes which contain $p60^{src}$ display phosphotransferaste activity.

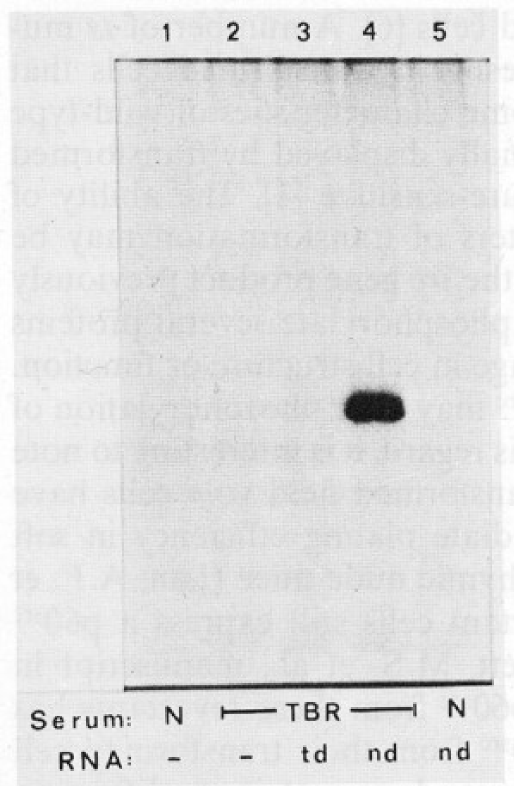

Fig. 3. Autoradiogram of SDS-polyacrylamide gel analysis of phosphorylated proteins. A portion of the bacteria-bound immune complexes obtained after protein synthesis and immunoprecipitation was resuspended directly in 25 μl of protein kinase reaction-mixture (Table 2). The phosphorylation of IgG was determined by SDS-polyacrylamide gel electrophoresis as described (Table 2, reference [4]).

C. Discussion

The results that we have presented in this communication and elsewhere demonstrate that a product of the ASV *src* gene is a phosphoprotein of molecular weight 60000 and that this protein appears to have protein kinase activity. Biosynthetic radiolabeling enables detection of only one transformation-specific polypeptide, $p60^{src}$, in the avian and mammalian cells transformed by ASV in which we have observed immunoprecipitable protein kinase activity. However, the possibility does exist that a cellular kinase specifically associates with $p60^{src}$ and is responsible for the observed enzymactic activity. This seems unlikely since such a cellular kinase would have to be present in both avian and mammalian cells as well as in reticulocyte cell-free extracts. Furthermore, additional experiments (M.S. Collett, unpublished data) support the association of the protein kinase activity with $p60^{src}$. The enzymatic activity and $p60^{src}$ cosediment during glycerol gradient centrifugation and coelute from ion exchange columns and immunoaffinity columns. The purification of the enzyme from cells infected with nondefective or *ts* virus will more clearly resolve these questions.

The fact that expression of protein phosphorylating activity is thermosensitive in *ts* mutant-infected cells suggests that the $p60^{src}$-associated protein kinase is directly involved in the transformation process and that ASV may transform cells by aberrant phosphorylation of cellular proteins.

In view of the well-documented role of protein phosphorylation in the regulation of cellular processes [14], this activity alone may be sufficient to initiate and maintain neoplastic transformation. Such a function of the transforming gene product would serve to explain many of the observations made

concerning the behavior of ASV-transformed cells [6]. A number of *ts* mutants of ASV have been described which result in transformed cells that display at the nonpermissive temperature some characteristics of wild-type transformed cells while other functions normally displayed by transformed cells are absent and thus are still temperature-sensitive [1]. The ability of these mutants to dissociate certain parameters of transformation may be related to the pleiotropic cellular response to the *src* gene product previously described [6]. The $p60^{src}$ protein kinase may phosphorylate several proteins and each in turn may produce a unique change in cell structure or function. Mutations occurring at different sites in $p60^{src}$ may alter phosphorylation of one cellular target but not that of others. In this regard, it is interesting to note that morphological revertants of SR-ASV transformed field vole cells have been isolated [8] which still exhibit intermediate plating efficiency in soft agar and the ability to produce tumors in athymic nude mice (Lau, A. F. et al., manuscript in preparation). These revertant cells still express a $p60^{src}$ protein which is enzymatically active (Collett, M. S. et al., manuscript in preparation). However, peptide analysis of $p60^{src}$ from these revertants has revealed a slight change, relative to the $p60^{src}$ from their transformed cell counterparts, in either primary structure or secondary protein modification of a single peptide (Collett, M. S. et al., manuscript in preparation). Such an alteration in $p60^{src}$ may reduce its ability to phosphorylate a particular cellular target protein, consequently producing a partial reversion of the transformed phenotype. Alternatively, a mutation in one of the cellular target proteins may render it resistant to $p60^{src}$-mediated phosphorylation. Direct identification of the proteins in normal cells phosphorylated by $p60^{src}$ will serve to clarify these issues. Moreover, possible functions for the product of the ASV transforming gene other than protein kinase should also be investigated.

D. References

1. Becker, D., Kurth, R., Critchley, D., Friis, R., Bauer, H.: Distinguishable transformation-defective phenotypes among temperature-sensitive mutants of Rous sarcoma virus. J. Virol. **21**, 1042–1055 (1977)
2. Brugge, J.S., Erikson, R.L.: Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature **269**, 346–348 (1977)
3. Brugge, J.S., Erikson, E., Collett, M.S., Erikson, R.L.: Peptide analysis of the transformation-specific antigen from avian sarcoma virus-transformed cells. J. Virol. **26**, 773–782 (1978)
4. Collett, M.S., Erikson, R.L.: Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. USA **75**, 2021–2024 (1978)
5. Eisenman, R., Vogt, V.M., Diggelmann, H.: Synthesis of avian RNA tumor virus structural protein. Cold Spring Harbor Symp. Quant. Biol. **39**, 1067–1075 (1974)
6. Hanafusa, H.: Cell transformation by RNA tumor viruses. In: Comprehensive virology. Fraenkel-Conrat, H., Warner, R.R. (eds.), **10**, 401–483 (1977)
7. Kessler, S.W.: Rapid isolation of antigens from cells with a Staphylcoccal protein A-antibody adsorbent: Parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**, 1617–1624 (1975)
8. Krzyzek, R.A., Lau, A.F., Faras, A.J., Spector, D.H.: Post-transcriptional control of avian oncornavirus transforming gene sequences in mammalian cells. Nature **269**, 175–179 (1977)

9. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970)
10. Marczynska, B., Deinhardt, F., Schulien, J., Tischendorf, P., Smith, R. D.: Rous sarcoma virus-induced tumors in marmosets: Characteristics of tumor cells and host cell-virus interrelationships. J. Natl. Cancer Inst. **51**, 1255–1274 (1973)
11. Pelham, H. R. B., Jackson, R. J.: An efficient mRNA-dependent translation system from reticulocyte lysates Eur. J. Biochem. **67**, 247–256 (1976)
12. Purchio, A. F., Erikson, E., Erikson, R. L.: Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. Proc. Natl. Acad. Sci. USA **74**, 4661–4665 (1977)
13. Purchio, A. F., Erikson, E., Brugge, J. S., Erikson, R. L.: Identification of a polypeptide encoded by the avian sarcoma virus src gene. Proc. Natl. Acad. Sci. USA **75**, 1567–1571 (1978)
14. Rubin, C. S., Rosen, O. M.: Protein phosphorylation. Ann. Rev. Biochemistry **44**, 831–887 (1975)