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A cellular protein phosphorylated by the avian sarcoma virus transforming gene product is associated with ribonucleoprotein particles

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In chick embryo fibroblasts transformed by Rous sarcoma virus (RSV) the tyrosine phosphorylation of a cellular protein of 34 000 daltons mol. wt. (34 kd) is greatly enhanced; this was shown to be catalyzed by the phosphotransferase activity of RSV transforming protein pp60^{src}. We report here that in cytoplasmic extracts of both normal and transformed cells, in the presence of magnesium ions, the majority of the 34-kd protein is associated with large structures and that a fraction of 34 kd appears to be associated with ribonucleoprotein particles (RNPs). In addition, upon u.v. light cross-linking of RNA to protein in normal or transformed cells, an anti-34 kd serum immunoprecipitates RNA fragments of apparent low sequence complexity as detected by T1 fingerprint analysis. Our results indicate that the 34-kd protein may play a role in the cell at the level of RNPs.

Key words: RSV transformation/substrate pp60^{src}/RNPs/u.v. cross-linking

Introduction

Transformation of chicken embryo fibroblasts (CEF) by Rous sarcoma virus (RSV) is dependent on the synthesis of the transforming gene product pp60^{src} (Brugge and Erikson, 1977; Hanafusa, 1977; Vogt, 1977; Purchio *et al.*, 1978). A function of pp60^{src} is to catalyze the transfer of phosphate from ATP to tyrosine residues of some polypeptides (Collett and Erikson, 1978; Erikson *et al.*, 1979; Collett *et al.*, 1980). Recently, a cellular protein with a mol. wt. of ~34 000 daltons (34 kd) was shown to be phosphorylated *in vivo* in a transformation-dependent manner; *in vitro* studies have confirmed that this protein is a specific substrate for the tyrosine kinase activity associated with pp60^{src} (Erikson and Erikson, 1980; Erikson *et al.*, 1981). The 34-kd protein appears to be identical with the 36-kd and 39-kd proteins described by others (Radke and Martin, 1979; Radke *et al.*, 1980, Cooper and Hunter, 1982). This transformation-dependent phosphorylation at the level of the tyrosine residues was shown to occur concomitantly with the earliest morphological changes induced by RSV transformation, using temperature-sensitive transformation mutants (Radke and Martin, 1979). Among the different transformation parameters, the increase in plasminogen activator was shown to correlate best with the increase in phosphorylation of 34 kd (Nakamura and Weber, 1982). These data do not eliminate, however, the possibility that pp60^{src} may have other functions (and obviously other substrates) in the transformation process. The implication of 34 kd in transformation by avian oncoviruses makes particularly relevant a study of its cellular location and function(s). Earlier reports (Erikson and Erikson, 1980) indicated that 34 kd was found mainly in the cytoplasm of either normal or transformed CEF. We report here a detailed analysis of the cellular location of 34 kd. We found that in both normal and transformed CEF, in the presence of magnesium ions, the majority of 34 kd is associated with large structures and that a fraction of it behaves similarly to proteins that are constituents of ribonucleoprotein particles (RNP). In addition, following u.v. light cross-linking of RNA to protein in normal or transformed CEF, an anti-34 kd serum immunoprecipitates RNA fragments of apparent low sequence complexity. Implications of these results for the transformation process are discussed.

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Results

Distribution of 34 kd in cytoplasmic extracts

Analysis by sucrose gradient sedimentation. Normal or transformed CEF were labeled overnight with [^{3}H]uridine and for 3 h with [^{35}S]methionine and then lysed in the presence of detergents as described in Materials and methods. The 16 000 *g* supernatant (containing $MgCl_2$) was treated as follows: aliquots were either incubated as such for 30 min at 0°C, or in the presence of 10 mM EDTA (final concentration) or incubated in the presence of 50 μg/ml RNase A plus or minus 10 mM EDTA. Each aliquot was then analyzed by sedimentation through sucrose gradients as described in Materials and methods. In the untreated aliquot, polysomes, monosomes and ribosomal subunits were resolved and four pools of fractions were selected according to their sedimentation coefficients: (a) >90S; (b) 60-90S; (c) 20-60S and (d) <20S. Each pool was then divided into three parts and proteins were respectively, TCA precipitated, immunoprecipitated with preimmune serum or with anti-34 kd serum and analyzed by electrophoresis on polyacrylamide gels. The results presented in Figure 1A show that 34 kd is found in every pool but in different amounts and a quantitative estimation is given in Table I: in the presence of 10 mM $MgCl_2$ both in normal or transformed CEF, ~2/3 of 34 kd sediments faster than 20S but very little is found in the fractions between 60 to 90S. Similar results are obtained when the distribution of 34 kd is based on the Coomassie blue staining of the protein or, in the case of transformed cells labeled for 2 h with ^{32}P, on the phosphorylation of 34 kd. Treatment with RNase A shifts 34 kd to the 0-20S pool suggesting a polysomal origin for part of the cytoplasmic 34 kd (not shown). In the presence of EDTA, both polysomes and monosomes are dissociated and 80% of the 34 kd is now found in the 0-20S pool, whereas 20% still sediments in the 20-60S pool (Figure 1B). Additional experiments indicate that 34 kd present in the 0-20S pool sediments slower than 15S. In the presence of EDTA and RNase, both RNPs and ribosomes are destroyed and 34 kd is now found only in the pool <20S (Figure 1C). To rule out possible unspecific binding of 34 kd to RNPs or ribosomes, the following control experiment was carried out: the top fractions of a sucrose gradient (see Figure 1A) containing free ^{35}S-labeled 34 kd were mixed with a 16 000 *g* cytoplasmic supernatant obtained

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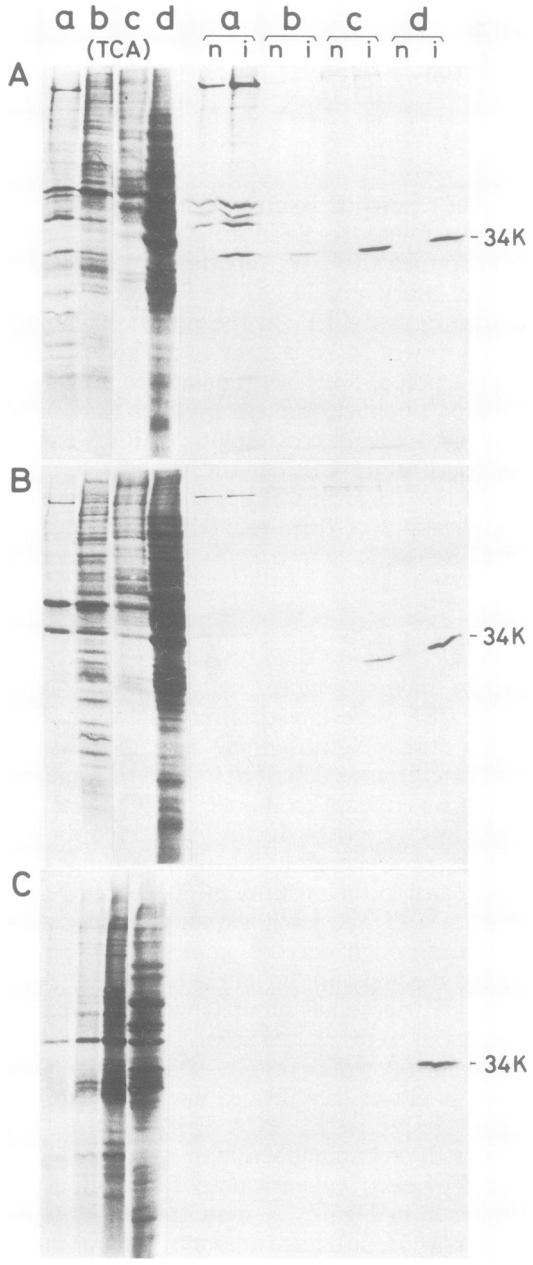

Fig. 1. Presence of 34 kd in polysomes and RNPs. CEF were labeled overnight with [^{3}H]uridine and then for 3 h with [^{35}S]methionine. Cells were lysed as described in Materials and methods and the 16 000 *g* supernatant was divided into three parts incubated as follows: **(A)** 30 min at 0°C, **(B)** same as **(A)** but in the presence of 10 mM EDTA (final concentration), **(C)** as **(B)** but in the presence of 50 μg/ml RNase A (heat-inactivated 5 min at 95°C) for 30 min at 37°C. Centrifugation in sucrose gradients was performed as in Materials and methods with the exception that **A** was spun in a gradient containing 10 mM EDTA in the gradients. Fractions of the different gradients were pooled in the following size range: >90S **(a)** 60–90S **(b)** 20–60S **(c)** and <20S **(d)**. A RIPA-SDS wash of the bottom of the centrifugation tube was included in pool **(a)**. Pools were divided into three parts and either immunoprecipitated (under conditions of antibody excess) with preimmune **(track n)** or anti-34 kd serum **(track i)** or TCA precipitated **(track TCA)**. Proteins were analyzed on 10% polyacrylamide gels as described in Materials and methods. Fluorographs of the gels are presented. The TCA tracks of experiments **A** (**c** and **d**), **B** (**c** and **d**) and **C** (**d**) were underexposed in order to show details of the bands.

from the same amount of unlabeled cells and analyzed in the presence of $MgCl_2$ as described above. Under these conditions labeled 34 kd remains at the top of the gradient (not shown) indicating that free 34 kd does not bind unspecifically

Table I. Distribution of 34 kd in cytoplasmic extracts

Cells	% 34 kd in the various fractions from the gradient			
	>90S	60–90S	20–60S	<20S
Normal	28	3	35	34
Transformed	25	3	33	39

Preparation of the various cytoplasmic extracts (containing 10 mM $MgCl_2$) and conditions of the sucrose gradient analysis are described in Materials and methods. Immunoprecipitation of the different fractions were performed (in antibody excess) as described in the legend to Figure 1. The immunoprecipitates were analyzed on 10% polyacrylamide gels and appropriate bands were excised from the gels and their radioactivity was determined in a liquid scintillation spectrometer. Radioactivity of equivalent areas in the preimmune tracks were subtracted from the values obtained with the immune serum. Results are given as percentage of the total cytoplasmic content of 34 kd in CEF labeled 3 h at 35°C with [^{35}S]methionine.

to an excess of RNPs, ribosomes and polysomes. These results indicate that in cytoplasmic extracts in the presence of magnesium ions the majority of the 34-kd protein is associated with structures sedimenting faster than the free protein. Also, based on S values and sensitivity of EDTA and RNase, several 34 kd containing structures can be defined: (1) one sediments faster than 90S and is EDTA and RNase sensitive, (2) one sediments between 20 and 60S and is partly EDTA resistant but RNase sensitive, and (3) one sediments slower than 20S.

Since two of the above structures containing 34 kd sediment in the same range as RNPs, ribosomal subunits and polysomes, they were further analyzed by density gradient centrifugation.

Analysis by metrizamide density gradient centrifugation. In metrizamide density gradients most of the proteins, polysomes and ribosomes band between 1.35 and 1.25 g/cm^3, RNPs at ~1.18–1.2 g/cm^3 and nucleic acids at ~1.15 g/cm^3 (Rickwood, 1976). We found that free 34 kd bands like the majority of the cellular proteins.

The polysomal fraction (>90S) was further analyzed in metrizamide density gradients, in the presence of 10 mM $MgCl_2$ or 10 mM EDTA, as described in Materials and methods. Results are presented in Figure 2 where it can be seen, in the presence of $MgCl_2$ or EDTA, that ^{35}S- and ^{3}H-labeled materials are recovered in two distinct peaks (1.3/cm^3 and ~1.18 g/cm^3). Immunoprecipitation of the two pools defined in Figure 2, shows that in the presence of $MgCl_2$, 80% of 34 kd bands at densities corresponding to polysomes and 20% at densities corresponding to RNPs. Note that at the density of polysomes, some ribosomal proteins stick to the immune complex. Following addition of EDTA to the >90S fraction, 34 kd is released and sediments in the 0–15S range. Metrizamide density gradient analysis of the >90S pool in the presence of EDTA is presented in Figure 2B where it is seen that 60% of 34 kd present in this fraction bands as a free protein and 40% as RNP complexes.

The same analysis in metrizamide density gradients performed with the 20–60S region of the sucrose gradient (containing $MgCl_2$) indicates that 34 kd (35% of total cytoplasmic 34 kd, see Table I) is essentially recovered at a density of ~1.18 g/cm^3 typical for RNPs. Upon addition of EDTA about half of the 34 kd present in the RNP region is released and bands as a free protein (not shown).

Similarly, metrizamide density gradient analysis of the 0–20S fraction of the cytoplasmic extract revealed that

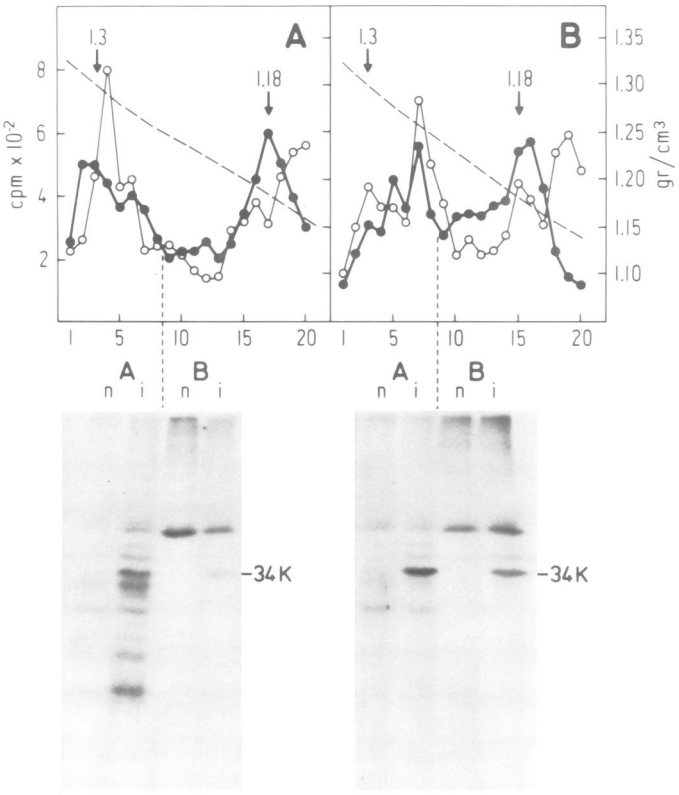

Fig. 2. Analysis of the >90S region in metrizamide density gradients. CEF were labeled overnight with [^{35}S]methionine and for the last 30 min with [^{3}H]uridine. Cells were lysed and the 16 000 *g* cytoplasmic supernatant was centrifuged in presence of $MgCl_2$ (see legend to Figure 1A). The 90S pool was divided into two parts. In one, $MgCl_2$ was added to a concentration of 10 mM and in the other EDTA was added to the same final concentration. Both preparations were mixed with a stock solution of 56% metrizamide to obtain a final concentration of 36% metrizamide. Centrifugation was performed as described in Materials and methods. **(A)** In the presence of $MgCl_2$. **(B)** In the presence of EDTA. (——) density. (—○—○—) [^{3}H]-uridine TCA precipitated counts, (—●—●—) [^{35}S]methionine TCA precipitated counts. In each gradient, pool A (1.35 – 1.25 g/cm^3) and pool B (1.25 – 1.15 g/cm^3) were defined. Each pool was divided into two parts and immunoprecipitated either with non-immune **(track n)** or anti-34 kd serum **(track i)**. Proteins were analysed on a 10% polyacrylamide gel as described in Materials and methods.

about half of the 34 kd bands at densities below 1.25 g/cm^3 in the presence or absence of EDTA (not shown). This suggests that about half of the 34 kd in the 0 – 20S fraction is of RNP origin.

Presence of 34 kd in the nuclear fraction

Normal or transformed CEF were labeled for 1 h with [^{35}S]methionine and the nuclear fraction was prepared as described in Materials and methods. As non-histone proteins are specifically extracted from chromatin by 0.35 – 0.4 M NaCl (Rabbani *et al.*, 1978) the nuclear pellet was extracted for 16 h with increasing NaCl concentrations up to 0.5 M and 34 kd was detected by immunoprecipitation. Results (not shown) indicated that 34 kd was gradually extracted by NaCl until a plateau value was reached with 0.5 M NaCl. The degree of contamination of the nuclear fraction by soluble cytoplasmic 34 kd during the preparation of nuclei was estimated by a mixing experiment: ^{35}S-labeled cytoplasmic and 0.4 M NaCl nuclear extracts were prepared, and the radioactive cytoplasmic extract was used to lyse the same amount of non-labeled cells; a 0.4 M NaCl nuclear extract from these cells was then prepared. 34 kd present in the two

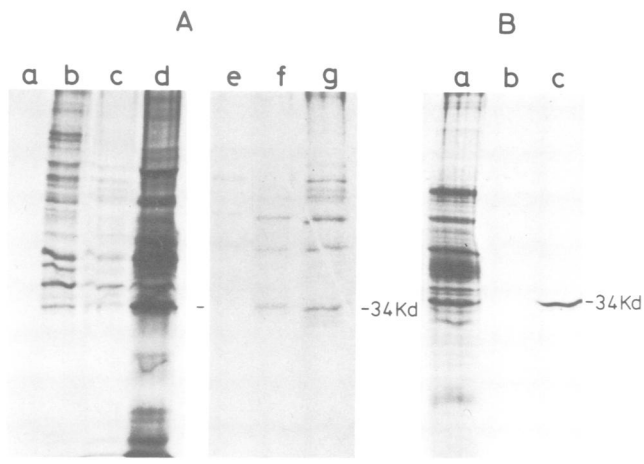

Fig. 3. (A) Proteins released from the nuclear fraction by RNase A treatment. The results of two independent experiments are presented. CEF were labeled for 3 h with [^{35}S]methionine and the nuclear pellet was isolated as described in Materials and methods. Crude nuclei were resuspended in 10 mM Tris pH 7.2, 10 mM EDTA at O.D. ~1 at 260 nm/ml and incubated at 0°C or 37°C for 30 min in the presence or absence of 10 μg/ml of RNase A (heat inactivated 5 min at 95°C to destroy possible DNase contaminant activities). After centrifugation for 15 min at 16 000 *g*, the supernatants were precipitated with 30% cold TCA (final concentration) and analyzed on a 10% polyacrylamide gel. Experiment (I) extracts **(a)** 0°C, **(b)** 37°C, **(c)** 0°C + RNase, **(d)** 37°C + RNase. Experiment (II) extracts: **(e)** 0°C, **(f)** 0°C + RNase, **(g)** 37°C + RNase. **(B)** Presence of 34 kd in the RNase A extract of the nuclear fraction. The RNase A extract of nuclei at 37°C was divided into three parts: one was precipitated with 30% cold TCA (final concentration) **(a)**. The two others were immunoprecipitated with either non-immune **(b)** or anti-34 kd **(c)** serum. Proteins were analyzed in a 10% polyacrylamide gel as described in Materials and methods.

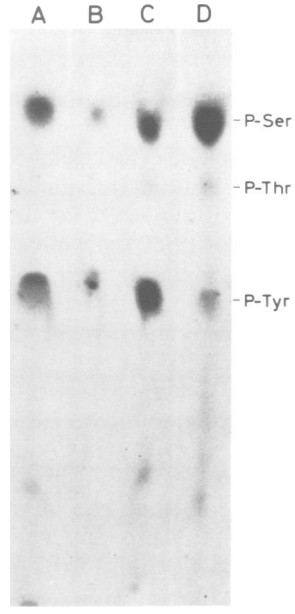

Fig. 4. Phosphoamino acids of 34 kd in different subcellular fractions. SRD-transformed cells were labeled for 2 h with ^{32}P and the 0 – 20S, 20 – 60S, >90S pools and the RNase A extract of the nuclear fraction were prepared. Each subcellular fraction was divided into two parts: one was immunoprecipitated with non-immune serum and the other with anti-34 kd serum. Analysis of the phosphoamino acid content of 34 kd excised from SDS gels was performed as described in Materials and methods. An autoradiography of the chromatogram is presented. **A**: 34 kd in the 0 – 20S pool; **B**: 34 kd in the 20 – 60S pool; **C**: 34 kd in the >90S pool; **D**: 34 kd in the RNase A extract of nuclei.

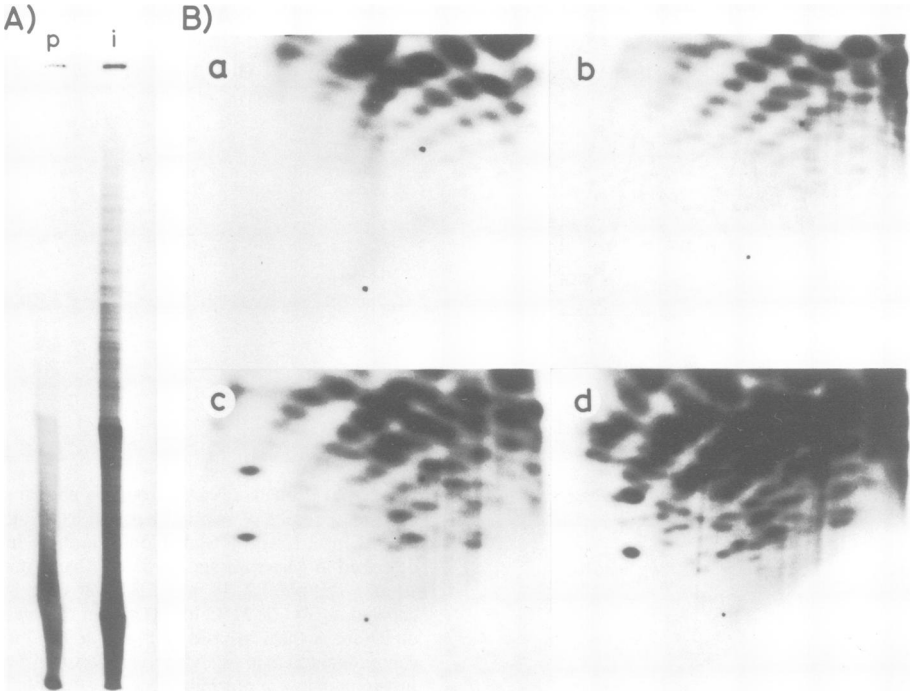

Fig. 5. Analysis of RNA u.v. cross-linked to 34 kd. **(A)** Polyacrylamide gel electrophoresis of RNA associated with 34 kd purified by immunoprecipitation. Cells were irradiated with u.v. light, lysed in hot SDS, and the lysates were immunoprecipitaed with either non-immune **(p)** or anti-34 kd serum **(i)**; the immunoprecipitates were treated with hot phenol-urea, digested with proteinase K and the RNA was ^{32}P-labeled as described in Materials and methods. RNA was analyzed on polyacrylamide gel in 7 M urea. Material at the bottom of the gel is 10 nucleotides long and the size of the strong band in the middle is 25 residues in length (1 h autoradiography using XS5 Kodak film). **(B)** Fingerprint analysis of RNA associated with 34 kd purified by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The RNA samples were essentially prepared as described above except that the proteinase K digestion step was omitted and 34 kd, as well as the corresponding band in the non-immune track, were isolated from an SDS-protein gel. The RNA present was digested with T1 RNase, labeled with ^{32}P and analyzed in two dimensions as described in Materials and methods. As a control experiment, HSA and anti-HSA were added to the lysate of u.v.-irradiated normal or transformed cells and the RNA non-specifically bound to the immune complex was purified and analyzed. **(a)** control experiment, HSA-associated RNA. **(b)** RNA present in the non-immune track (transformed cells, similar result is obtained in the case of normal cells). **(c)** 34 kd-associated RNA in normal CEF. **(d)** 34 kd-associated RNA in transformed CEF.

nuclear extracts was detected by immunoprecipitation and the results show that the degree of contamination is <1%. Although we cannot exclude cytoskeletal contaminations, the results presented above indicate that nuclear 34 kd represents ~10–15% of the total cellular 34 kd. In cells transformed by Schmidt-Ruppin RSV (SRD), a similar result is obtained. Digestion of the nuclear fraction with DNase I under conditions that preferentially digest the template-active sequences of chromatin (Weisbrod and Weintraub, 1979) did not specifically release 34 kd. EDTA had no effect, but RNase A or a temperature of 37°C were very effective (Figure 3A and B). No ribosomal proteins were detected in the nuclear fraction or in its RNase A extract after Coomassie blue staining of the gels (not shown). Analysis of the nuclear fraction in metrizamide density gradients was also performed. The nuclear fraction was sonicated to obtain a fraction enriched in soluble chromatin as described by Arrigo *et al.* (1980), which was analyzed in metrizamide density gradient as described before. We found that most of 34 kd banded at densities similar to RNPs and chromatin (1.15–1.25 g/cm^3); only ~20% of 34 kd present in soluble chromatin was recovered at heavier densities corresponding to free proteins. When chromatin was dissociated with 2 M NaCl, 34 kd was no longer present at intermediate densities and behaved as a free protein. Together with the effect of RNase (Figure 3), these results suggest that 34 kd in nuclei is also associated to RNP structures.

Phosphoamino acid analysis of 34 kd in the different cellular fractions

Only ~10% of total cellular 34 kd is phosphorylated at tyrosine residues in SRD-transformed cells (Erikson and Erikson, 1980). It was of interest to know if 34 kd associated with RNPs also contained phosphotyrosine. SRD-transformed CEF were labeled with ^{32}P as described in Materials and methods and polysomes, ribosomes and RNPs were prepared as described in Figure 1A. The nuclear pellet was then treated with RNase A as described in Figure 4. 34 kd was immunoprecipitated from the different fractions and phosphoamino acids analysis was performed as described in Materials and methods. The results, presented in Figure 4, show that in every fraction tested 34 kd contained phosphotyrosine, although no quantitative estimation can be made since we cannot exclude, in this type of analysis, some phosphatase activity.

U.v. light cross-linking of 34 kd to RNA in situ

In view of the possible association of 34 kd with RNPs we have used another approach that allows detection of protein-nucleic acid interactions. This technique, protein-nucleic acid cross-linking using u.v. light, has been used successfully to detect RNA or DNA proteins interactions in viruses and subcellular structures (Möller and Brimacombe, 1975; Wagenmakers *et al.*, 1980). Both normal and transformed CEF labeled with [^{35}S]methionine were irradiated with u.v. light,

lysed in hot SDS and 34 kd was recovered by immunoprecipitation as described in Materials and methods. We found that under these conditions of immunoprecipitation 34 kd (defined as a transformation-specific phosphoprotein) is still normally recognized by the serum. The immunoprecipitates were then treated as follows: a part was extracted with hot phenol-urea and the organic phase and the interphase were recovered, as under these conditions only those nucleic acids in protein-nucleic acid complexes are obtained (Wagenmakers *et al.*, 1980; J.-L.Darlix, unpublished data). Recovered RNAs (see Materials and methods) were labeled *in vitro* with [γ-^{32}P]ATP and electrophoresed on polyacrylamide gels in the presence of 7 M urea (see Materials and methods). It is apparent from the results shown in Figure 5A that anti-34 kd serum allows the detection of RNAs present as protein-nucleic acid complexes (Figure 5Ai) that are not observed in the control experiment (Figure 5Ap). This RNA material is of small size due to the methods of isolation and thus represents not more than the RNA sequences protected by the protein. Although the anti-34 kd serum immunoprecipitates essentially 34 kd in our conditions (see for instance Figure 1 and Discussion) it is nevertheless necessary under the conditions of u.v. cross-linking used here to show that the RNAs in the immune complex are indeed cross-linked to the 34-kd protein. Therefore, the second part of the immunoprecipitates, extracted with hot phenol urea, was resolved by SDS-polyacrylamide gel electrophoresis. The band of ^{35}S-labeled 34 kd and the corresponding one in the preimmune track were excised, and upon elution the materials were treated with proteinase K. The RNA present was purified by phenol extraction (see Materials and methods), digested with RNase T1 and the T1 oligonucleotides were labeled with ^{32}P at their 5′ ends and then resolved by two-dimensional gel electrophoresis (see Figure 5B (a – d)). To rule out a non-specific co-precipitation of RNA in the immune complex after u.v. cross-linking of cells, human serum albumin (HSA) and anti-HSA were added to the cell lysate of u.v.-treated normal and transformed cells. Fingerprint analysis of the contaminating RNA is presented in Figure 5Ba. Both in normal (Figure 5Bc) and transformed (Figure 5Bd) CEF, fingerprint of the RNA cross-linked to protein migrating at 34 kd displays two major unique T1 oligonucleotides (and some minor) not seen in the control analyses of the band in the preimmune track of the SDS gel (Figure 5Bb) or of the HSA/anti-HSA immune complex (Figure 5Ba). T1 fingerprint analysis of materials coming from two regions of the protein gel just above and below the 34 kd band, gave results identical to the controls (not shown). Thus, this experiment suggests that the T1 oligonucleotides detected should be specifically associated with 34 kd.

Discussion

The experiments reported here have made use of an antiserum directed against a purified 34-kd protein (Erikson *et al.*, 1981) to detect the presence of this protein in the various subcellular fractions. Whether this antiserum is entirely specific is not known but as far as the experiments on cellular localization are concerned, the immunoprecipitated material was always found to contain phosphotyrosine. Since no other cellular protein migrating in the 34 – 39 kd range has been found to be phosphorylated in tyrosine as a result of transformation by RSV this establishes that at least part of the immunoprecipitated material contains authentic 34 kd. This is sufficient to validate our data on the presence of 34 kd in the various subcellular fractions. In the case of the u.v. cross-linking experiments, whether only a single polypeptide is precipitated by the anti-34 kd serum is of course difficult to prove absolutely but several observations suggest that this is probably the case. First, the anti-34 kd serum has been obtained using a protein of rather high purity: upon two-dimensional gel analysis the immunoprecipitated material migrated as a single spot, be it ^{35}S- or ^{32}P-labeled (Erikson *et al.*, 1981). Second, when a quantitation of the material present in the 34-kd band immunoprecipitated from the various cytoplasmic fractions (Figure 1) is based on either ^{35}S or ^{32}P c.p.m. or on Coomassie blue staining, the same percentage of 34 kd distribution is found; in addition, the ratio P-Ser/P-Tyr in the immunoprecipitated material is the same in all these fractions and the quantitation of 34 kd in those fractions gives the same results whether it is based on ^{35}S or on phosphotyrosine content. Third, tryptic fingerprints of the ^{35}S-labeled immunoprecipitated material from the various cellular fractions are practically identical and consistent with there being only one protein of 34 kd (6 – 7 spots characteristic of authentic 34-kd protein). In addition, ^{32}P-labeled immunoprecipitated material displays the same tryptic fingerprint pattern as published for 34 kd (Erikson *et al.*, 1981). Thus, it is rather improbable that another polypeptide of 34 kd mol. wt. is present in the immunoprecipitated material together with authentic 34 kd (defined by the transformation-specific phosphoprotein).

How can phosphorylation of cellular proteins by pp60^{src} contribute to the transformation phenotype? To answer this question it is important to know what is the function(s) of the cellular substrates of pp60^{src}, and how phosphorylation by pp60^{src} could alter their function(s). As an approach to define the function(s) of the major substrate of pp60^{src}, i.e., 34 kd, we have undertaken a detailed analysis of its cellular location in both normal and transformed CEF. Our results show that ~85% of 34 kd is cytoplasmic, the remaining 15% being found in the nuclear fraction. By means of sedimentation analysis of cytoplasmic extracts under various conditions, we find that the majority (~2/3) of 34 kd, labeled either with ^{35}S, ^{32}P or Coomassie stained, sediments faster than 20S and is associated, in a magnesium-dependent way, with very large structures (>90S). Chelation of magnesium ions by EDTA destroys this association and allows us to distinguish two classes of 34 kd: one, which contains the majority of 34 kd and sediments slower than 20S and the other still associated with structures sedimenting between 20 and 60S. Since it is known that chelation of magnesium ions dissociate polysomes into ribosomal subunits and mRNA (Sherton *et al.*, 1974), the release from large structures of 34 kd together with a reduction of the sedimentation coefficient to ~ 10S suggest that this protein was bound to polysomes, not as a conventional mRNP but either free or associated with small RNPs. This was confirmed by metrizamide density gradient analysis of the polysomal fraction: in the presence of magnesium, 34 kd bands at the level of polysomes and RNPs, and upon addition of EDTA, 34 kd bands as a free protein and as small RNPs. On the other hand, the association of 34 kd with structures sedimenting between 20 and 60S which are resistant to EDTA argues in favour of the association of 34 kd with large RNP and further experiments, in particular analysis in metrizamide density gradients, support this

conclusion. It is not, however, known whether this association is through a direct interaction of 34 kd with large RNPs or is mediated by a small RNP. Our findings are in agreement with those of Cooper and Hunter (1982), who describe the association of a 39-kd protein (believed to be the equivalent to 34 kd described here) with large structures in a magnesium-dependent way. Finally, we find that 34 kd sedimenting slower than 20S is not only present as a free protein but half of it bands at the density of small RNPs in metrizamide density gradients. It should be pointed out here that, due to our conditions of cell lysis, we cannot exclude that a fraction of 34 kd was present in membranes since most of these were destroyed by the detergents used, 2% Nonidet-40 (NP-40) and 0.5% sodium deoxycholate. As far as the 34 kd present in the nuclear fraction is concerned, we cannot exclude contamination by the cytoskeleton, although others (Cooper and Hunter, 1982) have concluded that 34 kd is not associated with cytoskeletal structures. DNase I or EDTA were not able to release 34 kd, whereas RNase A, NaCl or a temperature of 37°C released 34 kd from the nuclear fraction. 34 kd present in the nuclear fraction showed also an intermediate density (1.2 g/cm^3) in metrizamide gradients and this was found to be salt dependent, suggesting that the 34 kd present in nuclei is also associated with RNP structures.

In transformed cells we have found a roughly similar cellular distribution of 34 kd and phosphoamino acid analysis revealed that, in every cellular fraction including RNPs, this protein contains phosphotyrosine residues; this is important since Erikson and Erikson (1980) have shown that only ~10% of cellular 34 kd is phosphorylated in transformed cells. Thus, the phosphorylation of 34 kd does not result in a gross alteration of its cellular distribution.

Possible direct 34 kd-RNA interactions were studied by irradiation of intact cells with u.v. light followed by immunoprecipitation and purification of 34 kd-RNA complexes by phenol extraction and separation on polyacrylamide gels. The results obtained show that, both in normal and transformed cells, anti-34 kd serum immunoprecipitates RNA, and that the nucleotide sequences of this RNA are of low apparent complexity, since only two unique T1 oligonucleotides 11 and 16 residues in length are detected. Control experiments, performed either with preimmune serum or with the HSA/anti-HSA immune complex indicate that these two unique T1 oligonucleotides do not arise from non-specific binding of RNA to the immune complex. Thus, these experiments suggest that some RNA can be cross-linked to 34 kd and confirm our results on the association of this protein with RNP.

Small cytoplasmic RNP particles may, through their binding to mRNPs or polysomes (free or membrane-bound), regulate the transport of mRNA from nuclei to cytoplasm. A recent report (Walter and Blobel, 1982) described a class of these particles (Signal recognition particle) implicated in the process of protein translocation across the endoplasmic reticulum. Whether or not 34 kd is involved in small RNP function, and the consequences resulting from its phosphorylation by pp60^{src} in transformed cells, remain to be shown.

Materials and methods

Cells and viruses

CEF were prepared from 11-day old embryos (Spafas, Inc., Roanoke, IL and Lohman Tierzucht, GMBH, Cuxhaven, FRG). SRD was originally obtained from J. Wyke. Transformed chicken cell were used for experiments several passages after they had been infected.

Radiolabeling of cells

Cultures were labeled with 50–100 μCi/ml [^{35}S]methionine (700 Ci/mmol; Amersham International) for different times in methionine-free medium or with 1 mCi [^{32}P]orthophosphate/ml (carrier-free, ICN, Irvine, CA) for 2 h in phosphate-free medium. Cells were also labeled with 100 μCi/ml [methyl-^{3}H]-thymidine (42 Ci/mmol; R.C. Amersham International) for 16 h in normal growth medium or with 100–200 μCi/ml [^{3}H]uridine (25 Ci/mmol, R.C. Amersham International) for different periods of time in normal growth medium.

Sucrose gradient analysis of polysomes and ribosomal subunits

Normal or transformed CEF were lysed in 10 mM Tris, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$, 1 mg/ml heparin, 2% NP-40 and 0.05% sodium deoxycholate. The cells were homogenized 10–20 times and spun for 10 min at 16 000 *g*. The supernatant, treated or not with EDTA or RNase, was loaded onto sucrose gradients (0.5–1 M) containing 10 mM Tris pH 7.2, 50 mM KCl, 10 mM $MgCl_2$ or 10 mM EDTA. The gradients were centrifuged for 17 h at 24 000 r.p.m. as described by Palmiter (1974) in a SW40 Beckman rotor. A sample of each fraction was diluted with 10 volumes of water, the proteins were precipitated with 30% cold TCA and the radioactivity was determined by counting the samples on glass filters (GF/C, Whatman) in a liquid scintillator spectrometer.

Analysis of RNPs in metrizamide gradients

Pooled fractions from sucrose gradients were mixed with a stock solution of 56% metrizamide (Nyegaard and Co.) in 10 mM Tris pH 7.2 in order to obtain a final solution of 36% metrizamide in 5 ml. The preparation was centrifuged for 42 h at 35 000 r.p.m. in a Ti50 rotor at 2°C according to Rickwood and Birnie (1973), Rickwood *et al.* (1974). A sample of each fraction was precipitated with TCA (30% final) and the radioactivity determined by liquid scintillation spectrometry; another sample was used to determine the density of each fraction, and the remainder was used for immunoprecipitation or direct analysis of proteins.

Preparation of the nuclear pellet fraction

Fractions enriched in chromatin were prepared by the method of Hancock (1974). Cytoplasmic contamination was assessed by examining nuclei or globular chromatin under a light microscope.

Immunoprecipitation of protein from the various cell fractions

Antiserum prepared against the 34-kd protein (anti-34 kd serum) has been previously described (Erikson *et al.*, 1981). Cell fractions were diluted with 2 volumes of RIPA buffer (10 mM Tris pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), non-immune or immune rabbit serum was added to each sample and the immune complexes were collected by adsorption to protein-A containing *Staphylococcus aureus* (Kessler, 1975) or to protein A-Sepharose. The adsorbants were washed once with 1 M NaCl, 0.01 M Tris pH 7.2, 0.1% NP-40 and several times with RIPA buffer in which the SDS had been replaced with 1 M urea. Samples were resuspended in SDS gel sample buffer (0.07 M Tris pH 6.8, 11% glycerol, 0.003% bromophenol blue, 3% SDS, 5% β-mercaptoethanol) and heated to 95°C for 1 min.

Polyacrylamide gel analysis of proteins

Proteins were either immunoprecipitated as already described or precipitated with 20–30% cold TCA (final concentration) for 3 h at 0°C. Proteins were washed once with acetone, acetone-ether (1:1), and ether, dissolved in SDS gel sample buffer and heated to 95°C for 1 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). Gels were stained, destained and dried as previously described (Erikson *et al.*, 1977). In the case of the ^{35}S-labeled proteins, gels were fluorographed with sodium salicylate (Chamberlin, 1979). Dried gels were exposed to Dupont Cronex 4 X-ray film at −70°C. ^{32}P-labeled proteins were located by autoradiography with the aid of Dupont Lightning Plus intensifying screens.

Phosphoamino acid analysis of the 34-kd protein

After immunoprecipitation and analysis of the different cell fractions in SDS-polyacrylamide gels, the ^{32}P-labeled 34 kd was located by autoradiography. 34 kd was then excised and eluted from the wet gel. 100 μg of bovine serum albumin was added as carrier and the protein was precipitated with 20% TCA for 16 h at 4°C. Precipitated proteins were resuspended and dissolved in 50 μl 1 N NH_4OH and hydrolyzed in 6 N HCl for 4 h at 110°C. HCl was removed under reduced pressure and the samples were dissolved in 2 μl of H_2O containing cold phosphoserine, phosphothreonine and phosphotyrosine (2.5 mg/ml each). Samples were spotted on a cellulose sheet

(Polygram Cel 400) and run for 1 h at 2500 V in pH 3.5 buffer (pyridine, acetic acid, H_2O, 1:10:189). Cold phosphoamino acids were revealed by ninhydrin and the ^{32}P-labeled phosphoamino acids were located by autoradiography.

U.v. light cross-linking experiment

[^{35}S]methionine-labeled normal or transformed CEF grown in Petri dishes were irradiated with u.v. light (15 W germicidal lamp, 252 mm) for 5 min at 4°C (Wagenmakers *et al.*, 1980). Cells were lysed in a buffer containing 10 mM Tris pH 7.5, 10 mM EDTA, 0.8 M LiCl and 0.1% SDS and, after heating for 2 min at 100°C, the suspension was centrifuged at 16 000 *g* for 10 min. The supernatant was diluted 5-fold with water and the pH adjusted to 8.8. Immunoprecipitation with non-immune or anti-34 kd serum was carried out as described above. That the antiserum still recognizes 34 kd after this treatment has been ascertained and we found that the efficiency of such an immunoprecipitation is as good as if the cells are lysed at 0°C with 0.1% NP-40 (as judged from the ^{32}P signal at the level of 34 kd in both cases). The immunoprecipitates were eluted with 50 μl SDS sample buffer. The eluates were diluted with 1 ml of water and mixed with 1 ml of phenol-urea at 80°C. Aqueous and organic phases were separated upon addition of 1 ml of chloroform. The phenol phase (2 ml) was then extracted five times with 5 ml of 0.2 M sodium acetate at pH 5.6. The organic phase and the interphase were treated in two different ways. (A) After ethanol precipitation and digestion with proteinase K (1 mg/ml, 30 min at 37°C) RNA cross-linked to the immunoprecipitated proteins was recovered by ethanol precipitations and then dephosphorylated using calf intestine phosphatase. After one phenol chloroform extraction, the RNA was precipitated with ethanol, labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described (Darlix *et al.*, 1979), and analyzed by polyacrylamide gel electrophoresis (12%) in 7 M urea. (B) After precipitation with 4 volumes of acetone the organic phase and interphase were analyzed by polyacrylamide gel electrophoresis in SDS and the ^{35}S-labeled 34 kd was detected by autoradiography. The band was excised from the gel as well as the corresponding one in the preimmune track, digested extensively (1 mg/ml, overnight at 41°C) with proteinase K in 0.1% SDS and RNA was extracted with phenol and precipitated with ethanol. RNA associated with 34 kd and that corresponding to the band in the preimmune track were denatured and digested with RNase T1. T1 oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Darlix *et al.*, 1979) and resolved by two-dimensional gel electrophoresis as described by de Wachter and Fiers, 1972) except that second dimension was run in 50 mM Tris-borate pH 8.3.

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