Volume 10 Number 10 1982 Nucleic Acids Research

Detection of a cellular polypeptide associated with adenovirus-coded VA RNA using in vitro labeling of proteins cross-linked to RNA

Chris van Eekelen*, Henny Buijtels*, Tommy Linné+, Rolf Ohlsson+†, Lennart Philipson+ and Walther van Venrooij*

*Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands, and +Department of Microbiology, The Biomedical Center, University of Uppsala, Box 581, S-751 23 Uppsala, Sweden

Received 5 April 1982; Accepted 27 April 1982

ABSTRACT

Ultraviolet light induced RNA-protein cross-linking for identification of polypeptides interacting with RNA in intact cells (Wagenmakers et al. 1980), is limited by the intensity of the label in the proteins or in residual nucleotides remaining attached to the proteins after RNase treatment of the RNA-protein complexes. Here we report a method, where the cross-linked RNA-protein complexes are treated with RNase T1 and the T1-oligonucleotides covalently linked to the proteins are labeled in the 5' terminus using γ-^{32}P-ATP and T4 polynucleotide kinase. The cross-linked proteins can then readily be identified owing to the incorporated ^{32}P label. As examples, proteins associated with polyadenylated mRNA, hnRNA and adenoviral VA RNA were identified. A protein with a molecular weight of approximately 50,000 is found associated with adenovirus-coded VA RNA. This was confirmed by binding assays, in which labeled VAI RNA is incubated with proteins from uninfected and adenovirus infected HeLa cells immobilized on nitrocellulose sheets.

INTRODUCTION

Proteins interacting tightly with RNA can be covalently linked to the RNA by irradiation with ultraviolet light in vivo or in vitro (1-8).The in vivo cross-linking technique allowed the detection of proteins interacting with mRNA and hnRNA in HeLa and Ehrlich Acites tumour cells (2-5). Also cellular polypeptides cross-linked to adenoviral mRNA and hnRNA could be analyzed, taking advantage of the possibility to select covalently linked viral RNA-protein complexes by hybridization to immobilized adenovirus DNA (6,8). Detection of the cross-linked polypeptides was performed using two different approaches of incorporating radioactive label. Either the protein moiety of the RNA-protein complexes was labeled with ^{35}S methionine or the RNA part was labeled by incubating the cells in medium containing ^{3}H nucleosides. Using the latter procedure, the cross-linked proteins were detected via the label incorporated in the residual oligonucleotide attached to the protein after ribonuclease treatment of the isolated RNA-protein complexes. The

0305-1048/82/1010-3039$ 2.00/0

latter method is specific and excludes detection of non-specifically copurifying polypeptides. However, the sensitivity of the method is limiting, since only a few nucleotides remain attached to each polypeptide (4). Therefore, the method can only be employed for RNAs that can be labeled in vivo to high specific activities and can be isolated in large quantities. In order to circumvent this problem and to make the technique more applicable also for less abundant RNA species, we introduce the present in vitro labeling method. In this procedure the cross-linked proteins were labeled with ^{32}P via end-labeling of the oligonucleotide that remains attached after RNase T1 treatment of the isolated RNA-protein complexes.

MATERIALS AND METHODS

Isolation of RNA-protein complexes

HeLa cells were cultured and infected with adenovirus type 2 (3,6). Ultraviolet light induced cross-linking of RNA to protein was performed as described previously (2,3). Irradiation of intact cells was for 5 min at 0^oC with a radiation dose of 8000 J $m^{-2}min^{-1}$ at 254 nm. Polyadenylated mRNA and hnRNA or cross-linked mRNA-protein and hnRNA-protein complexes were isolated from cytoplasmic extracts and nuclear matrices, using oligo dT-cellulose, as described earlier(3,4,6). The isolation of VA RNA-protein complexes was performed as follows. $4x10^8$ HeLa cells were labeled from 14 to 18 h post infection using 2.5 µCi/ml of each of the following ^3H nucleosides: (2,5',8-^3H) adenosine, (5-^3H) cytidine, (5-^3H) guanosine and (5,6-^3H) uridine (Amersham International, specific activities 42 Ci/mmol, 30 Ci/mmol, 23 Ci/mmol and 42 Ci/mmol ,respectively). After UV irradiation, a cytoplasmic extract of the cells (18 h post infection) was prepared, SDS was added to 0.5% and the sample was heated to 90^oC for 2 min. NaCl was added to 0.5 M and polyadenylated RNA or RNA-protein complexes were removed by incubation with oligo dT-cellulose during 1 h at room temperature and subsequent centrifugation. The supernatant was adjusted to 50% formamide , 750 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% bovine serum albumin, 0.02% polyvinylpyrrolidone-40, 0.02% Ficoll 400, 0.5% SDS and 0.5% Triton X100 and incubated with 1.8 mg Hind III B-fragment of the adenoviral genome immobilized on 10 g (wet weight) of cyanogen bromide activated Sepharose (6). Hybridization was for 60 to 100 h at 42^oC. Then the DNA-Sepharose was washed extensively and the RNA and RNA-protein complexes hybridized were eluted following the method described previously for viral hnRNA-protein complexes (6).

Isolation and labeling of T1 oligonucleotide-protein complexes

RNA-protein complexes precipitated by addition of 3 volumes of ethanol after their elution from oligo dT-cellulose or DNA-Sepharose were pelleted (15 min 10,000 xg) and dissolved in a small volume of H_2O ($\pm$ 100 µl) containing 0.5% SDS to ensure solubilization. The SDS was subsequently removed by spinning the samples through 1.5 ml Sephadex G-50 coarse in a 2 ml syringe at 400 xg for 10 min. Before use the G-50 was equilibrated in 10 mM Tris-HCl pH 7.4 and prespun in the syringe for 5 min at 400 xg. The volume of the eluate was twice that of the initial volume applied. The SDS free eluate was then treated with RNase T1 for 2 h at 37°C (4U/µl). After digestion 20 µg of carrier protein was added and the solution was extracted with phenol/chloroform. After centrifugation the aqueous layer was removed and the organic layer and the interphase were washed 4 times with buffer (25 mM Tris-HCl pH 7.4, 25 mM NaCl) to remove all RNA fragments not cross-linked to protein. After addition of 2 volumes of ethanol the RNA-protein complexes and other proteins present in the organic layer and interphase were collected by centrifugation at 10,000 xg for 5 min. The resulting pellet was washed once with ethanol to remove remaining phenol, dried and dissolved again in 100 µl 0.5% SDS by heating to 90°C for 2 min. The SDS was removed over Sephadex again and the eluate was used in the in vitro labeling reaction.

In vitro 5' labeling of T1 oligonucleotide-protein complexes was performed under standard conditions (9) using T4 polynucleotide kinase (New England Nuclear, E.C. 2.7.1.78) and γ-^{32}P-ATP (Amersham International, spec.act. 3000 Ci/mmol) for 1 h at 37°C. After incubation the ^{32}P-label associated with proteins could be recovered from the interphase after phenol/chloroform extraction. This interphase together with the organic layer was washed three times and proteins were recovered by addition of two volumes of ethanol. The precipitated proteins were pelleted, dissolved in sample buffer and analyzed on 13% polyacrylamide gels (10).

Preparation of cell extracts for protein blotting

A hypotonic swelling method was used to prepare cytoplasmic extracts. Cells were resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol), left on ice for 5 min and disrupted in a Dounce homogenizer (7 strokes) followed by low speed centrifugation (1000 xg) to pellet the nuclei. The supernatant constituted the cytoplasmic fraction. The nuclei were resuspended in 3 ml of 0.25 M sucrose, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 50 mM $Na_2S_2O_5$, 0.33 M NaCl and sonicated for 3x30 seconds in ice. The sonicated material was centrifuged at 16,000

xg for 10 min. The supernatant was used as a nucleoplasmic fraction. To the pellet was added 3 ml of the same buffer but with a NaCl concentration of 1 M. The resuspended pellet was sonicated and centrifuged as above. This second supernatant was used as the chromatin fraction. Analysis of the different fractions after SDS-polyacrylamide gelelectrophoresis and protein staining revealed that the chromatin fraction contained as major components all the histone proteins. No histones could be detected in the nucleoplasmic fraction by staining.

Transfer of polypeptides to nitrocellulose paper and RNA binding

The protein blotting procedure was essentially as described by Bowen et al. (11). The transfer was controlled by measuring the amount of ^{35}S-methionine labeled adenovirus 2 virion proteins transferred onto the filter. In a typical experiment the recovery was for polypeptide II: 28%, III 21%, IIIa 56%, VI 62 %, VII not transferred, VIII 23%, IX 50% and X 60%.

In vivo ^{32}P labeled RNAs were isolated as described by Westin et al. (12). The purity of the RNA preparations was tested by two dimensional fingerprint analysis (13,18). ^{32}P labeled RNAs (50,000 cpm) were diluted in 20 ml of BB-buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.5% bovine serum albumin, 0.05% polyvinylphosphate, 0.05% Ficoll) and incubated together with the protein blots in plastic pouches. Incubation was for 1 h at 20^{o}C. The nitrocellulose sheets were washed in 5x50 ml BB-buffer. After drying at room temperature the sheets were exposed to x-ray film for 3 days.

RESULTS

In vitro labeling of proteins cross-linked to RNA

In figure 1 a general procedure is outlined for the isolation and labeling of proteins associated with specific RNA sequences. Experimental details of the procedure are described in the Materials and Methods section. In a first step cells are irradiated with UV light to cross-link RNA to its associated proteins in vivo. Subsequently the cells are fractionated and from a suitable fraction specific RNA and RNA-protein complexes can be isolated by preparative hybridization to an immobilized DNA probe. This was oligo dT-cellulose for isolation of poly A(+) mRNA-protein and hnRNA-protein complexes and the Sepharose linked Hind III B-fragment of ad2 DNA for the isolation of VA RNA-protein complexes. The isolated complexes are extensively treated with RNase T1 and incubated with γ-^{32}P-ATP and T4 polynucleotide kinase for 5' terminal labeling of the RNA fragments. The complexes isolated and labeled in this way are then analyzed on protein gels. The results of the analyses are shown in

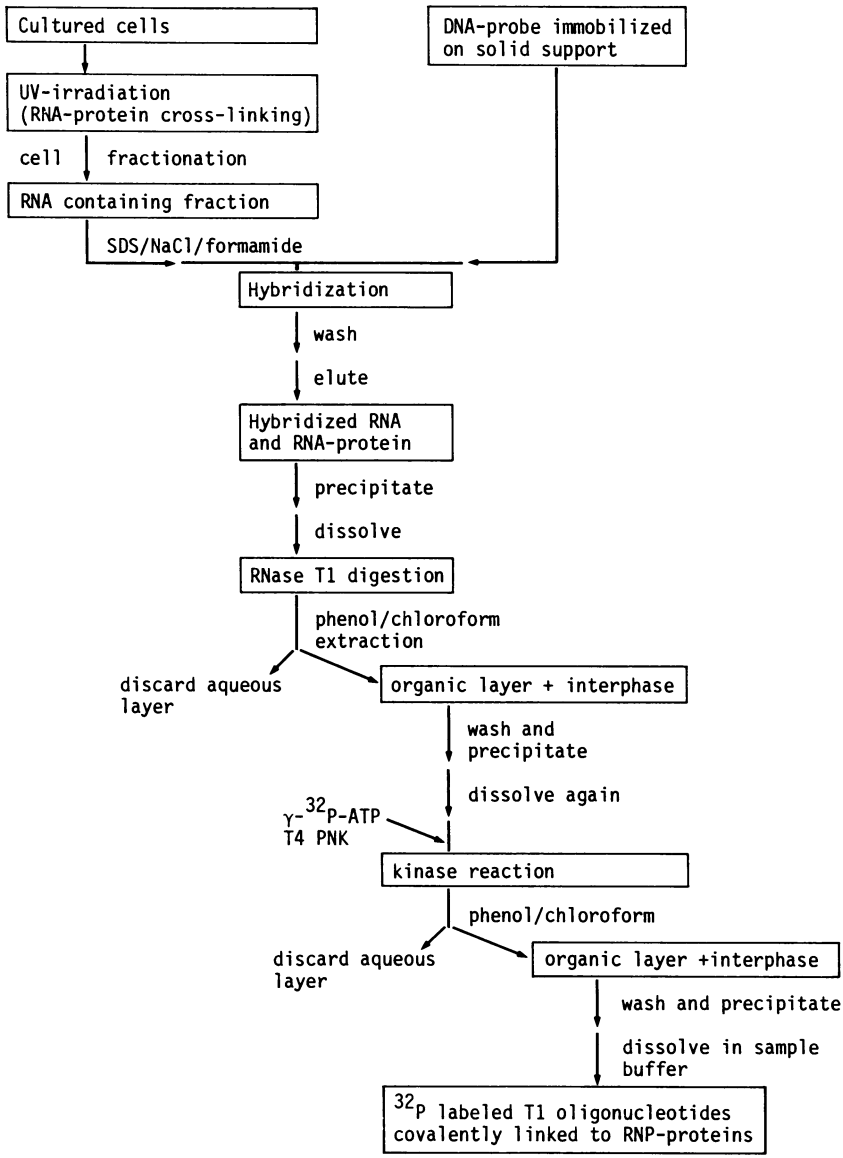


Fig.1 A general procedure for the isolation and labeling of proteins associated with specific RNAs.

fig. 2A for mRNA and hnRNA associated proteins. Fig. 2A, lane 2 shows the pattern of mRNA derived oligonucleotide-protein complexes, lane 3 of hnRNA derived oligonucleotide-protein complexes. In the lanes 1 and 4 the same

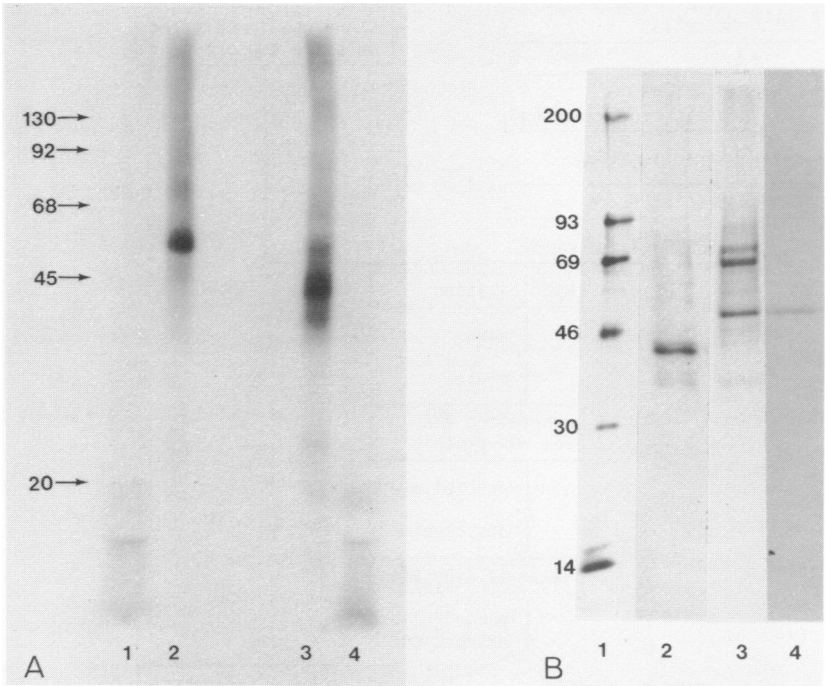


Fig.2. Analysis of proteins cross-linked to mRNA and hnRNA using in vitro ^{32}P labeling (3A) or in vivo ^{3}H labeling (3B). A. Covalently linked polyadenylated mRNA-protein and hnRNA-protein complexes were isolated from $4x10^{8}$ UV irradiated cells, as described (2-6). T1 oligonucleotide-protein complexes were isolated and the residual oligonucleotide part of the complexes were 5' end-labeled using γ-^{32}P-ATP and T4 polynucleotide kinase, as described in Materials and Methods (see also fig.1). The ^{32}P labeled T1 oligonucleotide-protein complexes were separated on a 13% SDS polyacrylamide gel. The gel was subsequently stained, dried and exposed to x-ray film for 2 h. The arrows indicate the positions of unlabeled marker proteins ($Mrx10^{-3}$). Lane 2: mRNA derived T1 oligonucleotide-protein complexes. Lane 3: hnRNA derived T1 oligonucleotide-protein complexes. Lane 1 as lane 2 and lane 4 as lane 3, after treatment of the samples with 100 µg/ml RNase A for 1 h at 37°C. B. The proteins cross-linked to polyadenylated mRNA and hnRNA (lanes 2 and 3) from $4x10^{8}$ UV irradiated HeLa cells, labeled in cell culture for 2 h with a mixture of all 4 ^{3}H nucleosides (0.5 mCi of each), were analyzed as described in previous reports (3,4,6). From the cytoplasmic material not bound to oligo dT-cellulose, non-polyadenylated mRNA-protein complexes were isolated using CsCl density gradients containing 0.5% Sarkosyl. The covalently linked mRNA-protein complexes containing fractions (densities from 1.35 to 1.45 g/cm^{3}) were collected, dialyzed and treated with RNases. The proteins with residual ^{3}H nucleotides attached, were precipitated by addition of 5% TCA and analyzed (lane 4).Lane 1: molecular weight marker proteins. Lane 2: proteins linked to poly A(+)hnRNA. Lane 3: proteins linked to poly A(+) mRNA. Lane 4: proteins linked to non-polyadenylated mRNA. The polypeptides in lanes 2-4 were detected by fluorography owing to the residual ^{3}H nucleotides attached to them. Exposure to x-ray film was for two months.

samples are analyzed but before application to the gel they were exhaustively treated with RNase A. The Mr range of the mRNA derived complexes is 53-57,000 dalton (mean 55,000). When the proteins cross-linked to mRNA were analyzed using in vivo labeling with ^{3}H nucleosides, we found polypeptides with molecular weights of 73,000 , 69,000 and 52,000 (fig. 2B lane 3), but in this case the contribution to the molecular weight of the residual RNA fragment was small (1-3 nucleotides). So the Mr 55,000 ^{32}P labeled band (fig.2A lane 2) probably corresponds to the 52,000 dalton polypeptide from the ^{3}H labeled picture (fig. 2B lane 3). The difference in molecular weight has to be ascribed to the T1 oligonucleotide. Apparently these oligonucleotides are heterogeneous in size as judged from the broad band on the ^{32}P autoradiograph. The 73,000 and 69,000 molecular weight polypeptides were not or very weakly found in the case of ^{32}P labeling. The explanation for this finding is probably that both these polypeptides interact directly with the poly A tail of mRNA. In accordance, proteins cross-linked to non-polyadenylated mRNA from uninfected HeLa cells using ^{3}H labeling, show only the 52,000 dalton polypeptide (fig. 2B lane 4)(courtesy of T.Riemen). It has been reported by many investigators that a polypeptide of Mr between 70 and 78 kilodalton is associated with poly A of mRNA (reviewed by Greenberg (14)). RNase T1 is not able to cleave poly A, so the proteins cross-linked to this part of the messenger will stay associated with large pieces of RNA throughout our isolation procedure. Furthermore, as cytoplasmic poly A tails are heterogeneous in length, the complexes will migrate in a broad Mr range higher than 70,000 dalton.

In the case of hnRNA a major band is found at 42-45,000 (mean 43,000) and bands of minor intensity at 38-39,000 and at about the same position as found for the mRNA associated polypeptides (fig. 2A lane 3). This latter band is most probably due to contamination with mRNA-protein complexes. In the comparable analysis using ^{3}H label (fig. 2B lane 2) we found two main bands at 41,500 and 43,000 and two minor bands at Mr 36,000 and 37,000, corresponding to the hnRNP C-proteins (main bands) and hnRNP B-proteins (minor bands), according to the nomenclature of Beyer et al. (15)(3,4,6). So also in this case there is a good correlation between the results found with in vivo ^{3}H and in vitro ^{32}P labeling, although, of course, in the latter case the apparent molecular weights of the RNA associated proteins are enlarged in average by 2,000 dalton due to the oligonucleotides attached to them. Although the ^{3}H autoradiograph of fig. 2B shows a superior resolution, the method using in vitro ^{32}P labeling has one main advantage: the sensitivity of detection has improved drastically. For comparison, fig. 2A is a photograph of a 2 h

exposure and fig. 2B of a 2 month exposure.In both experiments about the same number of HeLa cells was used to isolate the RNA-protein complexes. For the labeling in fig.2A 25 μCi of γ-^{32}P-ATP was used while in fig. 2B 2 mCi was added to the cells. The at least 100-fold increased sensitivity of detection enabled us to look at the proteins cross-linked to VA RNA.

Isolation of VA RNA-protein complexes

From a cytoplasmic extract of UV irradiated HeLa cells 18 h after infection with adenovirus type 2, VA RNA and VA RNA-protein complexes were isolated by preparative hybridization to the Hind III B-fragment of the viral genome, as described in Materials and Methods. To avoid contamination of the VA RNA preparation with viral mRNA, polyadenylated RNA was removed by binding to oligo dT-cellulose prior to hybridization. From the cytoplasmic poly A(–) RNA, labeled by incubation of the cells with ^{3}H nucleosides, 2.5 to 4% annealed to the DNA-Sepharose. Upon rehybridization 70% of the eluted RNA and RNA-protein complexes reannealed. Using phenol/chloroform extractions, in the way described by Wagenmakers et al. (2), about 10% of the RNA eluted from the DNA-Sepharose was found to be cross-linked to protein. Centrifugation of part of the eluate, after proteinase K treatment, on isokinetic glycerol gradients yielded a major peak sedimenting at 5.6 S (fig. 3A), corresponding to the sedimentation value for VA RNA. No RNA sedimenting faster than 8 S was found in the selected material. Analysis of the selected RNA on polyacrylamide-urea gel displayed a major RNA band with the mobility of VA RNA (fig. 3B lane 2). The eluate is highly enriched in this apparently undegraded VA RNA compared to the cytoplasmic extract (compare fig. 3B lanes 1 and 2).

In vitro labeling of VA RNA associated polypeptides

Notwithstanding using high amounts of ^{3}H nucleosides we were unable to detect proteins cross-linked to VA RNA due to the small amount of ^{3}H label attached to the VA RNA linked proteins. Using the in vitro labeling with ^{32}P the result shown in fig. 4 was obtained. Apart from two minor bands at 64 and 85 kilodaltons, a distinct major band at 50,000 is discernable as the main polypeptide cross-linked to VA RNA by in vivo UV irradiation.

The 50,000 Mr band is discrete as would be expected if the RNA part associated with this polypeptide is derived from only one species of RNA.

Identification of VA RNA binding polypeptides using protein blotting

To confirm that the 50,000 Mr band could bind specifically to VA RNA, we used the protein blotting technique. Cytoplasmic, nucleoplasmic and chromatin fractions from uninfected and adenovirus infected HeLa cells were separated by SDS-polyacrylamide gel electrophoresis (fig.5A). The polypeptides were

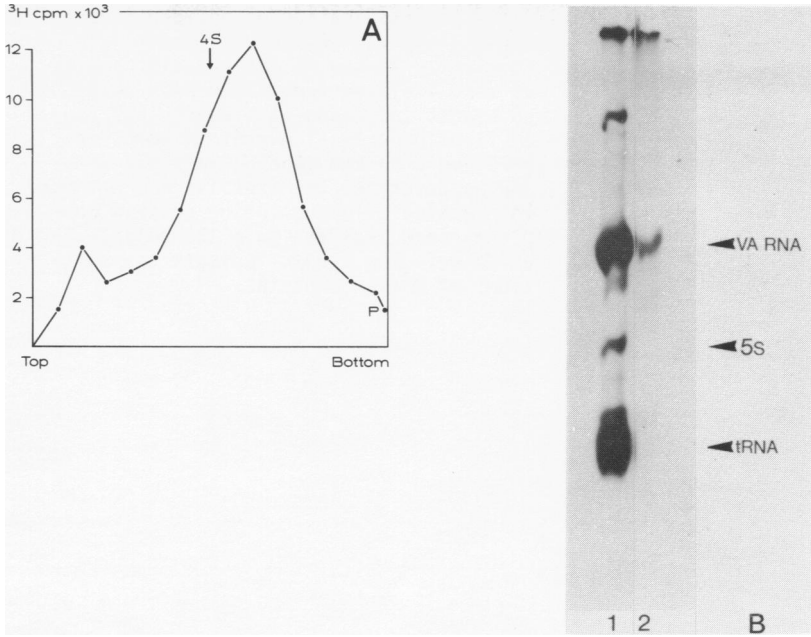


Fig.3. Characterization of non-polyadenylated RNA selected on the Hind III B-fragment of ad2 DNA. A. Glycerol gradient centrifugation. Centrifugation was for 18 h in a Beckman SW60 rotor at 40,000 rpm at room temperature on a 10-43% isokinetic glycerol gradient containing 10 mM Tris-HCl pH 7.4 and 0.1% SDS. The position of E.coli tRNA run on a parallel gradient is indicated. P indicates the amount of radioactivity pelleted. B. Analysis of selected RNA on a 10% polyacrylamide-urea gel. Lane 1: Total cytoplasmic RNA. lane 2: RNA eluted from the DNA-Sepharose.

transferred to nitrocellulose filters and probed with different snRNAs, VAI RNA and ribosomal RNA from both uninfected and infected cells. Fig. 5B shows that VAI RNA recognizes a polypeptide of 50,000 molecular weight, which is present both in cytoplasmic and nucleoplasmic extracts from uninfected and adenovirus infected cells harvested 6 and 24 after infection. In addition the VAI RNA probe strongly attaches to the histon H2a, H2b and H4 moieties, that are only present in the chromatin fractions. Fig. 5C shows that ^{32}P labeled ribosomal RNA under the same conditions can only recognize the histone proteins but no cytoplasmic protein. Similar experiments were also performed with 5S RNA and U2 RNA and the western blots in these cases revealed that the 50,000 dalton protein was only weakly if at all recognized by the 5S RNA and the U2 RNA. The difference in intensity on comparable blots between 5S and VAI RNA was more than 10 fold. These results suggest that the VAI RNA can

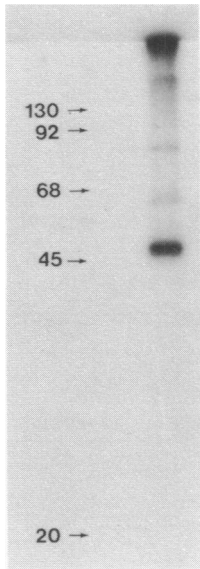


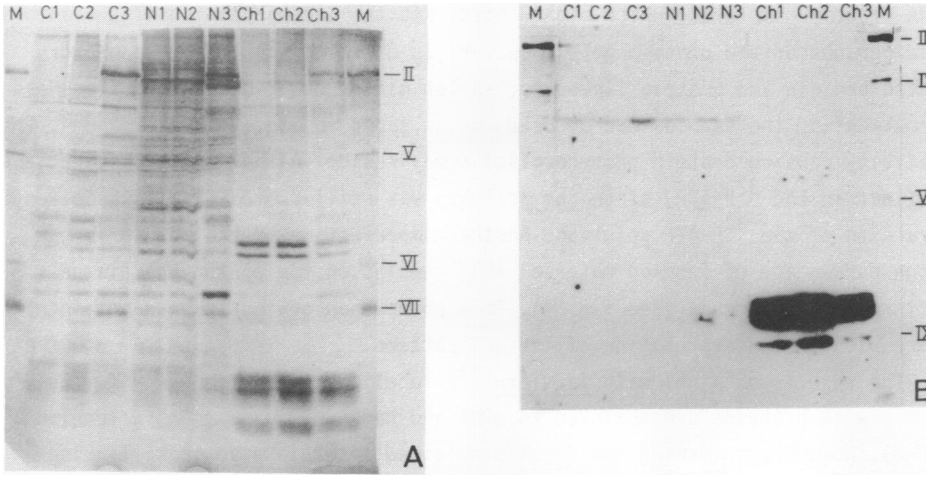
Fig.4. Identification of polypeptides cross-linked to VA RNA.
Covalently linked VA RNA-protein complexes were isolated from $4x10^8$ HeLa cells, 18 h post infection with adenovirus type 2. T1 oligonucleotide-protein complexes were isolated and labeled in vitro with ^{32}P as described in Materials and Methods. The labeled oligonucleotide-protein complexes were analyzed on a 13% polyacrylamide gel. The arrows indicate the positions of marker proteins.

interact with a cellular protein present in the nucleus and the cytoplasm, which is around 50,000 in molecular weight and corroborate the results from the cross-linking experiments.

DISCUSSION

RNA can be cross-linked to its associated proteins by *in vivo* (2-7) or *in vitro* (1,14) irradiation with UV light. The applicability of this technique for the identification of RNA associated proteins is of course limited by the possibilities to detect the cross-linked proteins. Until now, using ^{35}S methionine label of the protein or ^{3}H nucleoside label of the RNA, we have identified proteins associated with total cellular poly A(+) mRNA and hnRNA as well as adenovirus RNAs (2-8), but detection problems arose when we tried to identify VA RNA or snRNA associated proteins.

In this paper a method is introduced that increases the sensitivity of detection of RNA linked proteins by utilizing ^{32}P 5' terminal labeling of T1 oligonucleotide-protein complexes. It should be emphasized that, in principle, any nuclease generating 5'-OH groups can be used to label the proteins with this technique. We have in a separate study (17) used RNase A for the same purpose. It is of course also feasible to use enzymes generating 5'-P groups provided that an additional step with phosphatase treatment is introduced in the procedure. Using T1 oligonucleotides it was possible to confirm



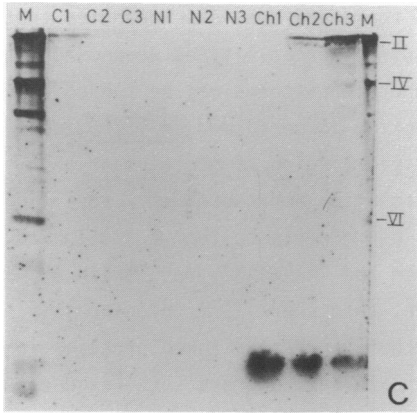


Fig.5. In vitro binding of VAI RNA to cellular proteins on nitrocellulose sheets. A. Stained pattern of a 13% SDS polyacrylamide gel containing different protein fractions. Cytoplasmic (C),Nucleoplasmic(N)and Chromatin (Ch) fractions from (1) uninfected HeLa cells, (2) early adenovirus and (3) late adenovirus infected cells. These protein fractions separated on gel as shown in A, were transferred to nitrocellulose sheets and incubated with purified RNA probes. B. Incubation with 50,000 ^{32}P cpm VAI RNA per sheet. C. Incubation with 50,000 cpm rRNA per sheet.As Mr markers served the adenoviral polypeptides II (120,000), IV (62,000), V (48,500), VI (42,000), VII (18,500) and IX (12,000), which were transferred together with the cellular proteins.

earlier findings concerning mRNA and hnRNA associated proteins. It was also possible to identify a polypeptide with an approximate molecular weight of 50,000 cross-linked to VA RNA.

This method to identify proteins interacting with RNA might have interesting possibilities in other studies of in vivo RNA-protein interaction. However, the method also has its pitfalls. It was found that during the in vitro labeling reaction using γ-^{32}P-ATP and T4 polynucleotide kinase phosphorylation of proteins may be observed. Labeled polynucleotide kinase as well as labeled RNase T1 were sometimes found on protein gels from control experiments without

RNA-protein complexes, which might complicate the interpretation of the autoradiographs of the protein gels. However, to discriminate between phosphorylated protein and protein linked to labeled oligonucleotides, extensive RNase treatment of the samples can be used, as is shown in fig. 2 lanes 1 and 4. The activity causing protein phosphorylation may reside in the enzyme preparations but not in the ^{32}P-ATP, since the activity was still present after phenol extraction of the ^{32}P-ATP solution. Another imperfection of the method is the high percentage of labeled material that remains on top of the protein gels after electrophoresis (see fig. 4). This phenomenon obviously does not interfere with the interpretation of the gel pattern.

The results obtained with in vitro ^{32}P labeling confirm our previous results with proteins cross-linked to mRNA and hnRNA using ^{3}H and ^{35}S labeling (2-6). However, comparing the results obtained after in vivo cross-linking with those of Setyono and Greenberg (16), obtained using in vitro cross-linking of ^{35}S methionine labeled polypeptides, there are some discrepancies that cannot be explained unambiguously. Although different cell types were used, it is our opinion that the differences in the protein patterns may be caused by differences in methodology. When UV cross-linking is performed in vitro, as done by Setyono and Greenberg (16), non-specific binding of proteins to exposed regions of the RNA during polyribosome isolation and subsequent cross-linking cannot be excluded. Furthermore , it is our experience that detection of cross-linked polypeptides by residual label in the RNA moiety results in a more specific and more simple protein pattern, than when ^{35}S methionine is used, due to contamination with co-purifying but non-cross-linked proteins in the latter case. In addition, ^{35}S methionine labeling can result in large differences in intensities for polypeptides isolated in equimolar amounts by variations in labeling kinetics or in methionine content of the polypeptides. Labeling the RNA results in more equal labeling efficiency irrespective of the nature of the polypeptide.

The finding that the major polypeptide that can be cross-linked to VA RNA has a molecular weight of approximately 50,000 is corroborated by the protein blotting experiments (fig. 5) showing that of the polypeptides of uninfected and early or late adenovirus infected cells only a similar sized host polypeptide, present in both nucleus and cytoplasm, was able to bind purified VA RNA.

Independently, other investigators have identified a host protein of similar molecular weight associated with VA RNA after synthesis in a cell-free transcription system (M.Francoeur and M.Matthews, Cold Spring Harbor

Lab., personal communication).

In conclusion our results point to a general method by which proteins cross-linked in vivo to specific RNA sequences can be identified, using cloned DNA probes for the isolation of the RNA-protein complexes and in vitro labeling for the detection of the cross-linked proteins. Furthermore, sequence analysis of oligonucleotides cross-linked to proteins may identify the part of the RNA molecule preferentially binding to the proteins. The localization of polypeptides on RNA molecules might help to identify the nucleoprotein structures involved in RNA splicing in mammalian cells.

ACKNOWLEDGEMENTS

We thank Dr.M.Francoeur and Dr.M.Matthews for communicating their results before publication. We are indebted to Dr.E.Mariman for help and advice. These studies were initiated during a stay of CvE at the Biomedical Center in Uppsala supported by an EMBO short term fellowship. The work in Nijmegen was supported by the Netherlands Foundation for Chemical Research and the Netherlands Organization for the Advancement of Pure Research and the work in Uppsala by grants from the Swedish Medical Research Council and the Swedish Association against Cancer. R.O. was the recipient of a post-doctoral fellowship from the Swedish Natural Science Research Council.

†Present address: Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden.

REFERENCES

1. Greenberg, J.R. (1979) Nucleic Acids Res. 6, 715-732.
2. Wagenmakers, A.J.M., Reinders, R.J. and van Venrooij, W.J. (1980) Eur.J. Biochem. 112, 323-330.
3. van Eekelen, C.A.G.and van Venrooij, W.J. (1981) J.Cell Biol. 88, 554-563.
4. van Eekelen, C.A.G., Riemen, T. and van Venrooij, W.J. (1981) FEBS letters 130, 223-226.
5. van Venrooij, W.J., Wagenmakers, A.J.M., van den Oetelaar, P. and Reinders, R.J. (1981) Molec.Biol.Rep. 7, 93-99.
6. van Eekelen, C.A.G., Mariman, E.C.M., Reinders, R.J. and van Venrooij, W.J. (1981) Eur.J.Biochem. 119, 461-467.
7. Mayrand, S., Setyono, B., Greenberg, J.R. and Pederson T. (1981) J.Cell Biol. 90, 380-384.
8. van Venrooij, W.J., Riemen, T. and van Eekelen, C.A.G. (1982) submitted.
9. Maxam, A.M. and Gilbert, W. (1980) in Methods in Enzymoloy, Vol.65,pp 499-560, Academic Press, New York.
10. Laemmli, U.K. (1970) Nature New Biology 227, 680-685.
11. Bowen, B., Steinberg, J., Laemmli, U.K. and Weintraub, H. (1980) Nucleic Acids Res. 8, 1-20.
12. Westin, G., Monstein, H-J., Zabielski, J., Philipson, L. and Petterson U.

(1981) Nucleic Acids Res. 9, 6323-6338.
13. Vennström, B., Petterson, U. and Philipson L. (1978) Nucleic Acids Res. 5, 195-204.
14. Greenberg, J.R. (1975) J.Cell Biol. 64, 269-288.
15. Beyer, A.L., Christensen, M.E., Walker, B.W. and LeStourgeon, W.M. (1977) Cell 11, 127-138.
16. Setyono, B. and Greenberg, J.R. (1981) Cell 24, 775-783.
17. Ohlsson, R., van Eekelen. C., and Philipson, L., submitted
18. Monstein, H-J. and Philipson, L. (1981) Nucleic Acids Res. 9, 4239-4250.