LOW MOLECULAR WEIGHT NUCLEAR RNA FROM SV40-TRANSFORMED WI38 CELLS; EFFECT ON TRANSCRIPTION OF WI38 CHROMATIN IN VITRO

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SUMMARY: Nuclei isolated from normal and SV40-transformed WI38 cells were used as templates for RNA synthesis in vitro. Comparison of template properties showed marked differences between the two chromatins with both homologous and heterologous enzymes. Addition of the 0.35 M NaCl extract from the chromatin of the SV40-transformed cells, revealed two separate activities: one stimulating the template activity of normal WI38 chromatin and one affecting the stability of the homologous RNA polymerase. Separation of the protein and SnRNA fractions in the extract demonstrated that the stabilization effect is due to chromosomal protein(s). However, SnRNA alone was found to be responsible for the stimulation of the transcriptional activity of normal cells to a level undistinguishable from that of the transformed cells.

Induction of malignant transformation of cells in culture by DNA and RNA tumor viruses, has been associated with the integration of viral genes or gene copies within the host cell genome (1). Such integration precedes the expression of some of the viral genes as well as an altered expression of the host genes. The mechanism of this viral-induced alteration in cellular gene expression has been the object of intense investigation in recent years. The implication of chromosomal proteins in this process has been supported by our previous finding of viral-induced modification in rates of synthesis phosphorylation and turnover of both histone and nonhistone chromosomal proteins in mouse and human cells transformed by SV40 viruses (2-11). While there appears to be some consistency in the effect of SV40-transformation of mouse and human cells with respect to the metabolism of chromosomal proteins, the significance of the observed changes remains to be established. Therefore, we decided to embark on a more functional approach to this study by comparing the template properties of chromatins from normal and SV40 transformed cells using both endogenous and E. coli RNA polymerases. This system was used to test the effect of addition of various components of chromatin. Work on chromosomal proteins in other laboratories has suggested that the 0.35 M NaCl extract from chromatin contains loosely-bound non-histone proteins with presumptive gene regulatory activity (12,13). However the presence in chromatin of small quantities of low molecular weight nuclear RNA (SnRNA) extractable at this salt concentration (14,18) has either been overlooked or purification methods have been inadequate to ensure an RNA-free protein preparation. It has been claimed that this RNA is intimately associ-

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ated with protein (19) and this could easily prevent its separation from the protein fraction whenever protein purification is carried out in nondenaturing conditions. More recently Marzluff et al (20), characterized these low molecular weight RNA's from mouse myeloma chromatin using improved methods for rapid and quantitative recovery of these species. The present investigation was designed to test the effect of the 0.35 M NaCl extract from chromatin of SV40-transformed cells on transcription of normal and SV40-transformed WI38 chromatin in vitro. Moreover, the extract was fractionated into its protein and SnRNA component in order to discriminate between the effects of each of the two macromolecular components.

MATERIALS AND METHODS: All experiments utilized log-phase cultures of SV40-transformed and normal WI38 human fibroblasts. Cell cycle analysis of both cell types revealed no significant differences in mean generation time (20 hrs) or length of "S"-phase (11±1 hr). Nuclei were isolated in ice-cold lysing medium containing 0.32 M Sucrose, 1% triton X-100 20 mM EDTA, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF), washed twice with 0.32 M sucrose, 0.01 M Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and either resuspended in 10% glycerol, 1% bovine serum albumin (BSA) or chromatin was isolated by lysis in ice-cold distilled water, allowed to swell for 30 min, homogenized gently and pelleted at 20,000 g for 20 min. Isolated chromatin was resuspended in 0.35 m NaCl, 0.02 M Tris-HCl pH 7.5 at a concentration of 1 mg DNA/ml, homogenized for 20 min. in an ice bath and centrifuged at 105,000 g for 2 hrs. Fractionation of RNA and proteins from the NaCl extract was achieved by either of the following procedures: A - the extract was dialysed against 200 volumes 1% SDS-0.1 M NaCl-0.01 M trisodium acetate - 1 mM EDTA (pH 5.4). The dialysate was incubated at 37°C for 15 min. and extracted with equal volumes of phenol and chloroform-isoamyl alcohol (34: 1 v/v). The aqueous phase was extracted twice more with chloroform-isoamyl alcohol, and the RNA precipitated with 3 volumes of ethanol and dried until use. B - the NaCl extract was dialysed against 200 volumes of 7 M urea - 0.25 M NaCl-0.05 M Tris HCl pH 5.5 and eluted through a DEAE-cellulose column until O.D 280 read 0. The column was washed with 0.3 M NaCl, 7 M urea, 0.05 M Tris-HCl pH 5.5 and RNA was eluted with 7 urea - 1 M NaCl in the same buffer (20). The proteins were collected, dialysed against 0.1 M Tris HCl pH 8.0 and lyophylised until use. The RNA fractions were collected, dialysed against 5 mM EDTA and lyophlized until use. For transcription assay isolated nuclei in 10% glycerol 1% BSA were incubated in either of the following assay mixtures: A - With E. coli RNA polymerase. Assay mixtures contained in 1 ml: 50 µmoles Tris-HCl pH 8.1, 3 µmoles Tris- HCl pH 8.1, 3 µmoles $MgCl_2$, 15 µmoles β-mercaptoethanol, 0.5 µmoles each ATP, CTP and GTP, 0.2 µmoles ^{3}H-UTP (75 µCi/ml) and 102 units E. coli RNA polymerase, isolated and purified as described (21). B - For endogenous polymerase assays the reaction mixtures were similar to the above except that no polymerase of Mg++ were added, and $(NH_4)_2SO_4$ was added to a concentration of 200 µmoles per ml.

Incubations were carried out at 25°C and reactions stopped by addition of 10 volumes ice-cold 10% TCA-40 mM sodium pyrophosphate; precipitates were collected at 8,000 g for 10 min. 50 mM NaOH was added to disolve each pellet and nucleic acids were again precipitated by addition of equal volumes of 10% TCA and collected on GF filters. THe filters were washed with TCA and ethanol, dried and counted in a Beckman liquid scintilation spectrometer. DNA was determined either by UV absorption or by the indole procedure (22) RNA by UV absorption (260 nm) and protein by Lowry (23) or O.D. 280.

For assay of initiation and elongation of RNA transcripts, incubation

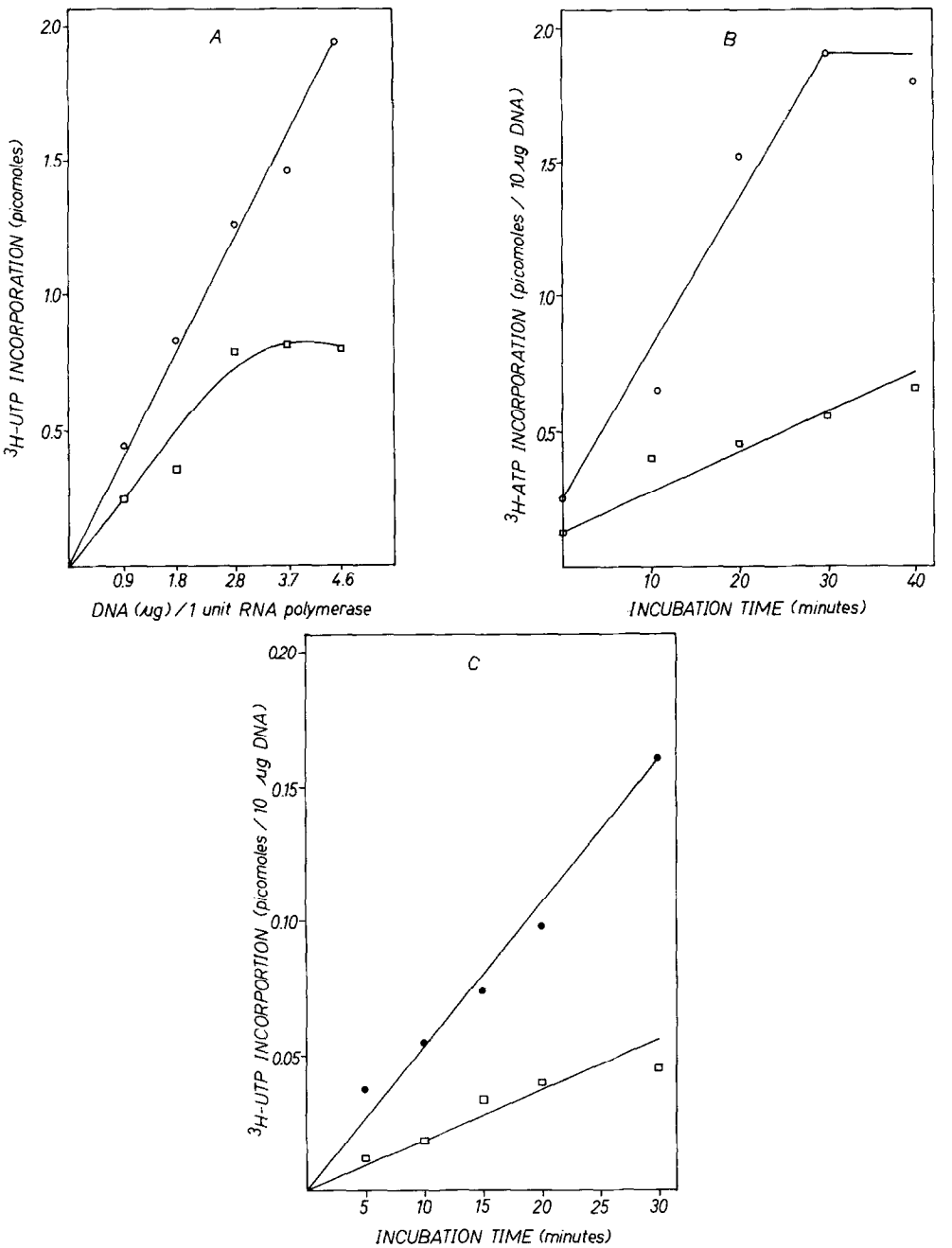

Fig. 1 Comparison of template activities of nuclei isolated from log-phase WI38 and SV40-transformed WI38 cells. A: with E. coli RNA polymerase at increasing DNA to enzyme ratios. □ nuclei from normal WI38 cells; ○ nuclei from SV40-WI38 cells. B: with 4.3 µg DNA per unit of E. coli RNA polymerase. Initiation was stoped after 14 min. and elongation allowed for 40 more min. as described under methods. □ nuclei from normal WI38 cells; ○ nuclei from SV40-WI38 cells. C: nuclei incubated under conditions optimized for endogenous RNA polymerase II activity. □ nuclei from normal WI38 cells; ○ nuclei from SV40-WI38 cells.

with E. coli polymerase was carried out as above but without CTP for 15 min, at which time $(NH_4)_2SO_4$ was added to a conc. of 0.4 M. This is required to prevent further initiation by RNA polymerase (24). At this time CTP was also added, to allow for RNA chain elongation, the incubation was carried out for various time periods and reaction was stopped as above.

RESULTS AND DISCUSSION: The template activity of nuclei isolated from log-phase WI38 or SV40-transformed WI38 cells was compared using either E. coli RNA polymerase or endogenous enzyme as described under methods. In Fig. 1A incorporation of ^{3}H-UTP into an acid insoluble product can be seen to increase linearly with increasing DNA in the case of SV40-transformed cell nuclei indicating that at the highest DNA/polymerase ratios there is still excess enzyme to cope with additional template. However, in the case of normal WI38 cells, not only the reaction appears to proceed at a lower rate but there is also a saturation level beyond which no further increase is seen. In order to test whether these differences can be attributed to number of initiation sites in chromatin, rate of elongation of RNA chains or both, we incubated both types of nuclei with E. coli RNA polymerase in the absence of CTP in order to allow initiation but to prevent elongation. Addition of CTP and salt allowed for elongation without further initiations (20) Fig. 2B. Zero time values indicate that there are more initiation sites in SV40-WI38 cell chromatin than in normal chromatin. In addition, the rate of chain elongation is much higher in transformed than in normal cell chromatin. When nuclei are incubated without addition of E. coli RNA polymerase, under conditions where endogenous RNA polymerase II is preferentially stimulated (25), the amount of ^{3}H-UTP incorporated is considerably lower (Fig. 2C). This is probably due to the low amounts of endogenous RNA polymerase retained in the nuclei isolated with the present procedure. However, the differences in the transcriptional activity of nuclei isolated from either normal or SV40-transformed WI38 cells are still apparent. It appears therefore that the heterologous E. coli polymerase is detecting genuine differences in template structure.

In subsequent experiments, the heterologous system was utilized for studying the effect of components of SV40-transformed WI38 cell chromatin on template activity of chromatin from both normal and transformed cells, while the endogenous system was utilized for comparison and as a test for possible RNA-polymerase-specific interactions. Preliminary experiments showed that the 0.35 M NaCl extract from the chromatin of SV40-transformed cells was capable of stimulating transcription in isolated WI38 nuclei with both exogenous and endogenous polymerase. However, it only stimulated, to a lesser extent, nuclei isolated from the same transformed cells whenever endogenous polymerase was tested (26,27). This suggested the existence in this extract of two separate activities, one affecting template structure of

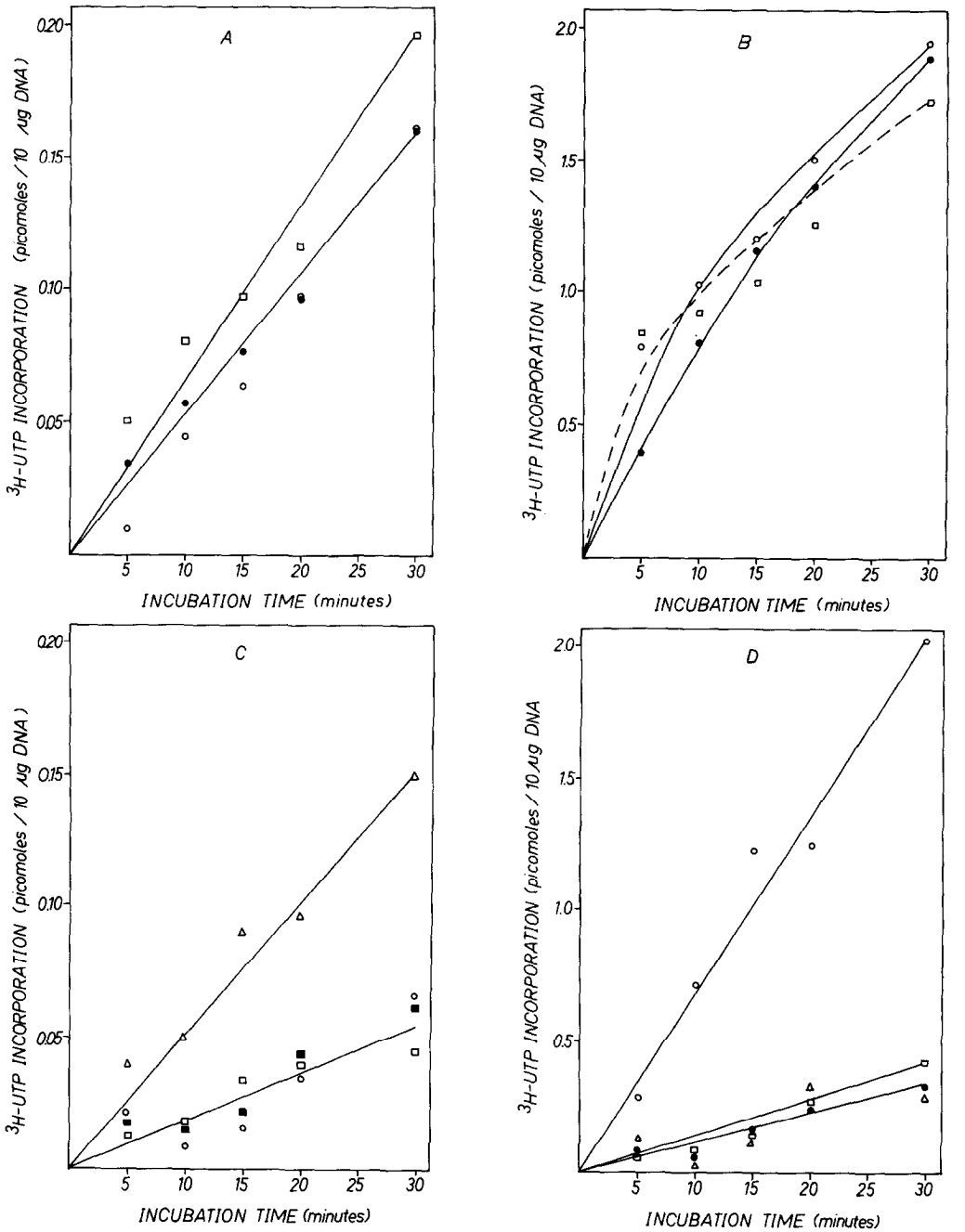

Fig. 2 Effect of addition of nonhistone chromosomal proteins (ℓ-NHCP) and SnRNA from the 0.35 M NaCl extract of SV40-WI38 chromatin. A- nuclei from SV40-WI38 incubated under conditions optimized for RNA polymerase II activity; ● control; □ with ℓ-NHCP in 1:1 w/w proportion to DNA; ○ with SnRNA in 0.1:1 w/w proportion to DNA; B-nuclei from SV40-WI38 cells incubated with E. coli RNA polymerase at 4.3 µg DNA/unit. ● control; □ with ℓNHCP in 1:1 w/w proportion to DNA; ○ with SnRNA in 0.1:1 w/w proportion

normal chromatin, the other affecting endogenous RNA polymerase. Since this extract was found to contain not only loosely-bound non-histone chromosomal proteins (1-NHCP) but also small amounts of tightly bound low molecular weight nuclear RNA (SnRNA), we separated the two fractions in order to test the effect of each on transcription *in vitro*. Fig. 2A illustrates the results in nuclei of the same SV40-transformed WI38 cells using the endogenous RNA polymerase incubation system. It is apparent that the SnRNA has no effect but that 1-NHCP addition has a stimulatory effect on ^{3}H-UTP incorporation. If, however, the same experiment is carried out with heterologous *E. coli* RNA polymerase no effect of either SnRNA or 1-NHCP could be detected (Fig. 2B).

These experiments were interpreted to mean that 1-NHCP from SV40-transformed WI38 chromatin contain a protein (or proteins) which have a polymerase-specific stimulatory activity on transcription. If these same 1-NHCP and SnRNA fractions are added to nuclei of normal WI38 cells incubated with either homologous (Fig. 2C) or heterologous (Fig. 2D) RNA polymerase, a similar stimulation of ^{3}H-UTP incorporation into RNA can be observed in both cases by addition of the SnRNA fraction from SV40-WI38 cell chromatin, while SnRNA purified in a similar manner from normal WI38 chromatin or 1-NHCP from SV40-WI38 chromatin show no stimulatory activity in the conditions of either Fig. 2C or Fig. 2D. In both cases the stimulatory effect of SnRNA appears to render the transcriptional activity of normal nuclei undistinguishable from that of transformed ones.

Even though the same results were obtained with SnRNA preparations purified by two different procedures, we considered the possibility that the preparations might contain some contaminating molecule which could be responsible for the activation effect. Since any molecule suspected of a regulatory role is likely to exist in only small quantities in the cell, the problem of accidental contaminants in any preparation is a serious one. If RNA is indeed the active molecule one must first prove that the effect is RNAse-sensitive, while ensuring removal of RNAse in the SnRNA preparation which would otherwise affect the RNA product during the transcription assay. The use of carboxymethylcellulose-bound RNAse prior to assay of the effect of SnRNA on transcription *in vitro* , provided the desired conditions.

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to DNA. C- nuclei from normal WI38 cells incubated under conditions optimized for RNA polymerase II activity. □ control, ■ with ℓNHCP in 1:1 w/w proportion to DNA, △ with SnRNA in 0.1:1 w/w proportion to DNA, ○ with the same amount of SnRNA isolated from normal WI38 chromatin. D-nuclei from normal WI38 cells incubated with *E. coli* RNA polymerase at 4.3 µg DNA/unit; □ control, ● with ℓ-NHCP in 1:1 w/w proportion to DNA, ○ with SnRNA in 0.1:1 proportion to DNA, △ with the same amount of SnRNA isolated from normal WI38 chromatin.

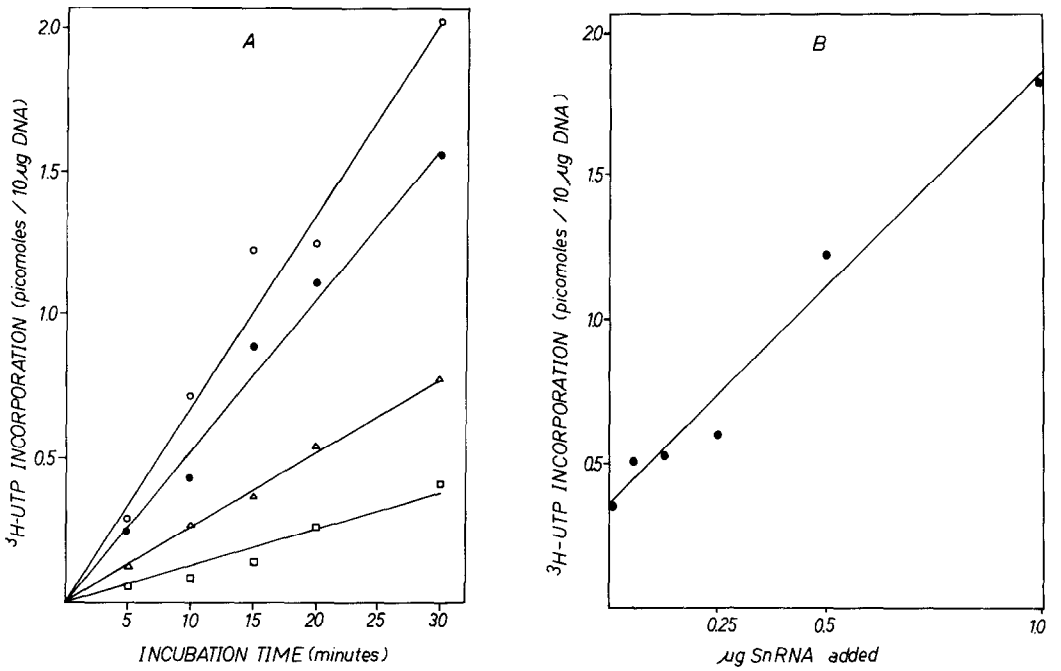

Fig. 3 Dose response curves for activity of SnRNA in stimulating transcription of WI38 chromatin in the presence of E. coli RNA polymerase at 4.3 µg DNA/unit. A-SnRNA was incubated with carboxymethyl cellulose-bound RNAse for either 1 or 3 hrs. under conditions which hydrolysed 50% and 90% of the RNA respectively. □ control, ○ with SnRNA in 0.1:1 w/w proportion to DNA, ● with SnRNA pretreated for 1 hr. with RNAse, △ with SnRNA pretreated for 3 hrs. with RNAse. B- effect of addition of different amounts of SnRNA.

The results are illustrated in Fig. 3A. Different incubation times with the bound RNAse were designed to eliminate the possibility that release of RNAse from the cellulose matrix, rather than specific digestion of SnRNA, might be responsible for the results. These data demonstrate that the "active" molecule in the SnRNA preparation is indeed RNA. Moreover the amounts of "active" SnRNA relative to DNA used in the transcription experiments appear to be in excess of those needed to demonstrate its stimulatory activity, as demonstrated in Fig. 3B.

The presence in chromatin of small quantities of low molecular weight nuclear RNA has been known for some time (14,18). However, because of the small quantities present and because of its close association with protein (19),it is difficult to isolate and purify in large enough quantities for transcription assays. Moreover, it is very sensitive to contaminating RNAse even when stored at -40°C, and since most RNAse inhibitors also interfere

with transcription, we found that not only we had to purify it very rapidly but also that it could only be stored after lyophilization.

Preliminary characterization using ^{32}P-labelled SnRNA separated by acrylamide gel electrophoresis and detected by autoradiography indicate that the "active" RNA is smaller than tRNA. We are currently investigating the possible role of these RNA's in transcription regulation in general and viral transformation in particular.

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