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Immunological evidence for the role of phosphoprotein p68/pI = 7.3 in premessenger RNA splicing

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A 68 kDa (pI = 7.3) nuclear phosphoprotein has been previously characterized as a component of transcriptionally active chromatin. Two-dimensional PAGE Western blotting and radioimmunoassay with monoclonal antibodies have identified this protein in nuclear extracts used for in vitro RNA splicing. In vitro splicing activity could be quantitatively inhibited by preincubating nuclear extracts with the antibodies, but the assembly of 60 S spliceosomes could not.

Phosphoprotein, nuclear; mRNA splicing, pre$^-$; Antibody; Spliceosome

1. INTRODUCTION

Nonhistone nuclear proteins have been widely studied for their potential role in gene regulation (see reviews [1–3]). A nuclear phosphoprotein has been previously identified within operationally defined transcriptionally-active chromatin and shown to become more abundant following 16 h of liver regeneration [4–6]. This purified protein has been used for preparing monoclonal antibodies [7]. Using these antibodies for two-dimensional PAGE Western blotting, this phosphoprotein was also identified among proteins which comprise the operationally defined nuclear matrix fraction [8]. Moreover, electron microscopy of immunogold-labeled thin sections revealed a preponderance of this nuclear protein in the vicinity of the nuclear envelope [9]. From these data, the phosphoprotein has been proposed to have a role in transcriptional or posttranscriptional processes.

In this report, we present data which demonstrate the presence of the phosphoprotein in nuclear extracts used for in vitro RNA splicing and show that monoclonal antibodies specific for this protein inhibit the ability of the extracts to carry out RNA splicing.

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2. MATERIALS AND METHODS

Nuclear extracts were prepared from spinner culture HeLa cells grown to mid log phase in RPMI 1640 media containing 10% fetal calf serum essentially as described by Dignam et al. [10]. Modifications included triethanolamine (TEO), pH 7.9, as a buffer, dithiothreitol (DTT) was omitted, and 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.05 μg/ml leupeptin and aprotinin and 20 U/ml soybean trypsin inhibitors were included in all buffers. Protein concentrations of the extracts averaged 6 mg/ml.

In vitro RNA splicing reactions were carried out under optimized conditions [11] with SP-6 adenovirus late-leader sequence single intron (IVS) transcripts [12,13] as splicing precursors. The IVS substrate (456 nucleotides) contained the natural first and second exons of the adenovirus late transcription unit separated by a shortened intron. Sliceosome assembly was determined by electrophoresing one-fifth of each in vitro splicing reaction into native, low porosity agarose-acrylamide composite gels [14].

Characterization of monoclonal antibodies and the radioimmunoassay were as described previously [7]. Western blotting conditions were also described previously [8,15] and utilized ^{125}I-protein A and autoradiographic detection system.

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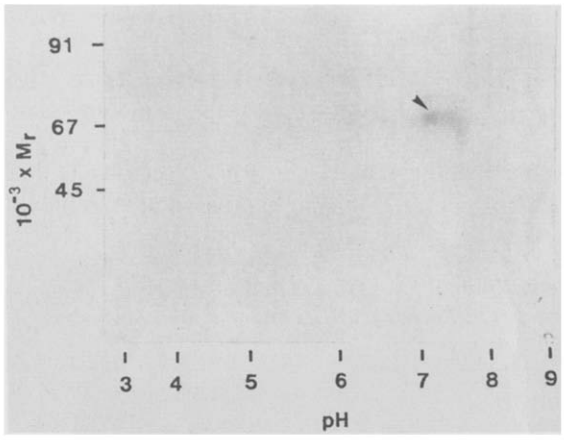

Fig.1. Nuclear extract proteins (80 μg) were resolved on two-dimensional gels, transferred to nitrocellulose and reacted with IgG_{2a} monoclonal antibody against the nuclear phosphoprotein (1:200 dilution). The M_r distribution and pH gradient are shown to the left and bottom of the figure, respectively.

3. RESULTS

Two-dimensional PAGE of nuclear extract proteins followed by Western blotting with anti-phosphoprotein IgG_{2a} monoclonal antibody revealed the presence of an antigen with appropriate size and isoelectric point, 68 kDa and pI = 7.3, respectively (fig.1). Radioimmunoassays confirmed these findings and demonstrated saturable (and therefore specific) antigen-antibody binding (table 1).

Nuclear extracts preincubated with IgG_{2a} monoclonal antibody on ice for 30 min were no longer competent for in vitro RNA splicing compared to controls (fig.2). Monoclonal antibodies

Table 1

Monoclonal antibody	Nuclear extract (μg)	Bound cpm
Nonimmune IgG	1	309
	10	176
	50	252
Immune IgG_{2a}	1	794
	1	794
	10	1453
	50	2300

Nonimmune IgG represents the tissue culture fluid. IgG_{2a} is the monoclonal antibody of a nuclear phosphoprotein. Radioimmunoassay was carried out by using a goat anti-mouse Ig-^{125}I

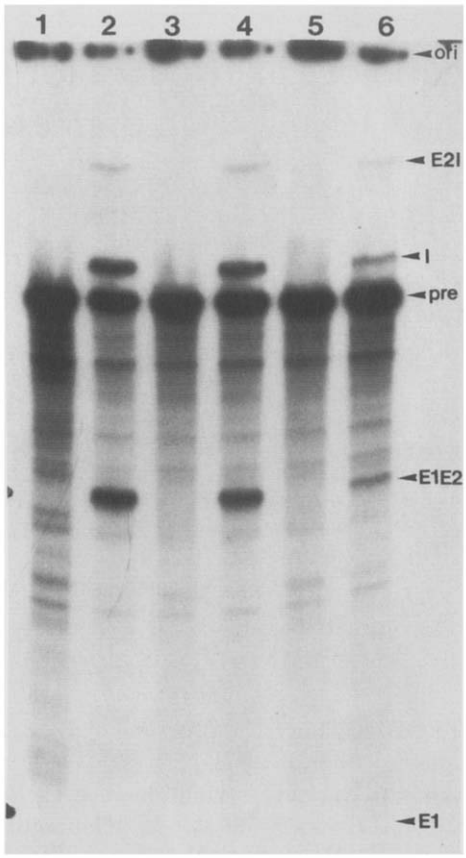

Fig.2. RNA was purified from in vitro splicing reactions, resolved by PAGE and autoradiographed. Radiolabeled RNAs corresponding to the splicing-precursor (premRNA), intermediate products intron-exon 2 (E2I) and exon 1 (E1), and products intron (I), exon 1-exon 2 (E1E2) are shown to the right. Lanes 1 and 2 correspond to control splicing reaction at time zero and after 60 min of incubation, respectively. Lanes 3–6 are all 60 min incubations which had been pretreated with 2 μl of native antibody, 2 μl of heat-inactivated antibody, 10 μl of native antibody, and 10 μl of heat-inactivated antibody, respectively.

reactive with UsnRNA common or unique proteins also inhibited splicing activity in the extract (data not shown and [21]). Boiling of the antibodies for 3 min prior to their addition to the extract uniformly prevented the antibody inhibition of RNA splicing. These findings suggest that the antibody effect was specific and that the phosphoprotein might have a role in RNA processing.

In vitro RNA processing involves an ordered assembly of the RNA splicing-precursor into a

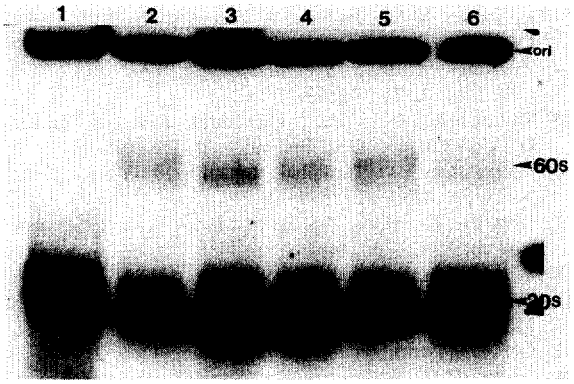

Fig.3. Ribonucleoprotein complexes within in vitro splicing reactions were resolved on native acrylamide-agarose gels and those containing radiolabeled RNAs visualized by autoradiography. The position of 20 S and 60 S complexes are indicated to the right. Lanes 1 and 2 correspond to complexes seen at time zero and after 60 min of in vitro reaction, respectively. Lanes 3–6 are complexes seen after 60 min of reaction which had been pretreated with 2 μl of native antibody, 2 μl of heat-inactivated antibody, 10 μl of native antibody, and 10 μl of heat-inactivated antibody, respectively.

60 S complex [11,14]. To determine at what stage in this process antibody inhibition occurred, complexes within the splicing reactions were resolved by native, agarose-acrylamide gel electrophoresis [14]. Immediately after adding the radiolabeled RNA splicing-precursor to the extract, 20 S nonspecific complexes form, which are transformed in a time-dependent fashion into functional 60 S spliceosomes [14]. Addition of the monoclonal antibody (with or without boiling) did not affect this assembly process (fig.3), whereas anti-UsnRNP antibodies block this process at the 20 S complex stage (data not shown and [21]).

4. DISCUSSION

Nonhistone nuclear proteins have been widely speculated upon as gene regulatory elements [1–3]. Monoclonal antibodies specific for a 68 kDa phosphoprotein [7] have been previously used to localize this protein within the ultrastructure of transcriptionally active nuclear domains [3–5,7–9]. From these studies, it has been proposed that this nuclear phosphoprotein may be involved in the anchorage of transcriptionally active genes to the nuclear matrix [6] and thereby possibly involved in transcriptional or post-transcriptional nuclear processes.

The data presented here demonstrate the presence of p68/p*I* = 7.3 in splicing-competent nuclear extracts, and suggest that the protein might be involved in premessenger RNA processing. The inhibition of splicing by native and not heat-inactivated IgG_{2a} suggests that the data reflect specific interference with p68's function by the antibodies. The ability of 60 S spliceosome complexes to assemble in the presence of the IgG supports the possibility that the data are not due to nonspecific effects. Moreover, assembly of 60 S complexes which are not competent for RNA splicing suggests that p68 might be involved in splicing during some stage following spliceosome assembly.

Proteins with molecular masses similar to the one described here have been reported among those UV cross-linkable to precursor RNA in vitro [16–18], as components of uridine-rich small nuclear ribonucleoprotein particles [19] and involved in early recognition of intervening sequences [20]. Most recently, we found that monoclonal antibodies specific for the 70 kDa U1-snRNP protein [22] failed to react the 68 kDa nuclear protein, which was isolated and purified from 2D gels (not shown), while at the same time the monoclonal antibody of the 68 kDa protein reacted strongly in a distinctive region at p*I* 7.3 as shown in fig.1. The possible relationship of these proteins to this nuclear protein reported here will be of interest in future studies.

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