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METHOD

Nuclease protection of RNAs containing site-specific labels: A rapid method for mapping RNA–protein interactions

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ABSTRACT

This report describes a method that combines nuclease protection and site-specific labeling to determine sites and extents of RNA–protein interactions. The utility of the method is demonstrated by the analysis of the binding of factors to the 3' splice site region of a pre-mRNA labeled at three specific positions. This “reverse footprinting” technique should be widely applicable to a variety of questions concerning RNA–protein interactions.

Keywords: nuclease protection; RNA–protein interactions; RNase H; site-specific labeling

INTRODUCTION

Nuclease protection analysis is a powerful technique for determining sites of RNA–protein interaction. A variety of protocols, colloquially termed “bind and chew,” have proven especially informative regarding snRNP/pre-mRNA contacts during pre-mRNA splicing (e.g., Mount et al., 1983; Chabot et al., 1985). In these approaches, labeled pre-mRNA is incubated with either purified components or crude extract under the desired conditions. The reaction is then quenched on ice and accessible portions of the pre-mRNA digested with one or more nucleases. Protected fragments are then recovered via immunoprecipitation (e.g., Mount et al., 1983; Chabot et al., 1985), or in some cases, directly visualized on denaturing gels (Ruskin & Green, 1985). The sequence content of the nuclease-resistant fragments is then determined by direct RNA analysis (i.e., fingerprinting).

Although straightforward in concept, these methodologies are not in wide use, chiefly because identification of protected fragments is viewed as cumbersome. In this regard, it is not trivial to determine unambiguously the RNase T1 map of a long pre-mRNA, and because fragment identification can be labor intensive,

it is not easy to use multiple substrates for comparative analyses. To overcome these difficulties, we have used pre-mRNAs containing site-specific labels for nuclease protection studies. The presence of the specific label “pre-identifies” protected fragments and avoids potential complications if multiple regions are protected. When combined with site-specific RNase H mediated cleavage (Lapham & Crothers, 1996; Lapham et al., 1997; Yu et al., 1997) of nuclease-resistant fragments, it is straightforward to rapidly, and with high precision, map sites of contact between proteins and/or snRNPs with labeled RNAs. We have previously used this method to examine interactions of snRNPs (U1 and U4/U6.U5 tri-snRNP) with the 5' splice site and recombinant U2AF with the 3' splice site (Wu et al., 1999; Maroney et al., 2000). Here, we provide an additional example by analyzing complexes formed at the branch point and 3' splice site. This technique, outlined schematically in Figure 1, is easily adapted to multiple substrates and, in principle, should be broadly applicable to a wide variety of questions pertaining to RNA–protein interactions.

METHODS

Preparation of site-specifically labeled pre-mRNA

We have found that the most convenient and quickest way to generate site-specific labels is to cleave tran-

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A. Site-specific labeling **B. Nuclease protection and mapping**

FIGURE 1. Schematic representation of the experimental strategy. In **A**, the straight arrow indicates the site of cleavage by RNase H (see Lapham et al., 1997). In the colored oligonucleotide, blue residues are 2' Ome and red residues are deoxy (adapted from Lapham et al., 1997). For details see the text.

scripts by site-specific digestion with RNase H (Lapham et al., 1997). Briefly, a single transcript (synthesized from a template with an optimized phage promoter) is trace labeled with $\alpha\text{-}^{32}\text{P}$ GTP to a specific activity of 40 cpm/ng. Approximately 10 μg is then annealed to a chimeric 2' Ome/DNA oligonucleotide and cleaved essentially as described by Yu et al. (1997); large scale digestions are for 1 h at 37°C with 10 U of RNase H (USB catalog number 27-0894-02, previously Pharmacia). Labeled half molecules are then separated on a denaturing gel and purified. The 3' half is treated with phosphatase and then labeled to high specific activity with polynucleotide kinase and $\gamma\text{-}^{32}\text{P}$ ATP (NEN catalog number NEG-035C).

After deproteinization, the labeled 3' half molecule is joined to the 5' half molecule using splinted ligation (Moore & Sharp, 1992). In our experience, the use of equimolar amounts of 3' half, 5' half, and bridge gives good yields ($\geq 90\%$) of ligated molecules and we have not yet found the need to manipulate ratios. In general, a 30-nt bridge oligonucleotide is used, although longer bridges may be required if the junction sequence is AU rich. For ligation, we have found that the rapid ligation kit (Roche Molecular Biochemicals) works well; ligations are carried out for 1 h at room temperature.

There are several advantages to using this labeling strategy as opposed to the more widely used method

of employing separate templates to synthesize the 5' and 3' half molecules. First, there is no need to construct two templates. Second, there exist no sequence constraints on placement of the label. In this regard, it is well established that the efficiency of in vitro transcription by phage polymerases depends critically on the first few nucleotides of the transcript (e.g., Milligan et al., 1987). Accordingly, some transcripts (e.g., those that begin with pyrimidines) can be difficult or impossible to synthesize with reasonable yield. Third, the real concern of having untemplated nucleotides appended to the 3' end of the 5' transcript is eliminated. Fourth, the molar yield of half molecules is equivalent, eliminating the need to adjust ratios for subsequent ligation. Finally, chimeric 2' Ome/DNA oligonucleotides are relatively inexpensive and are readily available commercially.

Nuclease protection assays

An example of a nuclease protection assay is shown in Figure 2. Here, we examined association of factors with the 3' splice site region of a pre-mRNA that serves as a substrate for spliced-leader addition in an *Ascaris* extract (Hannon et al., 1990). The pre-mRNA was specifically labeled at three different positions: (1) near the branch point (−25), (2) within the polypyrimidine tract

FIGURE 2. Nuclease protection of site-specifically labeled pre-mRNA. A *trans*-splice acceptor pre-mRNA, indicated schematically, was labeled at positions -25 , -6 , or -1 using site-specific cleavage of the pre-mRNA (see text). The sequence of the RNA from -36 to $+3$ is 5'-AUGAGUAGCGUUGAAUAAGUUGUUCGUUUUUUAG/GUA-3', where the slash indicates the intron exon boundary. Cleavage oligonucleotides were UUCAdAdCdGdCUACUCAU (BP), AAAAdAdAdTdACGAACAAC (PY), and UACcdTAdAdAAAAUACG (AG); bridge oligonucleotides used for ligation were 30-mers complementary to 15 nt on either side of the ligation junction. Individually labeled pre-mRNAs were incubated with *Ascaris* extract under splicing conditions for 0 (lanes T_0) or 15 min either in the absence (lanes $-$) or presence (lanes U2) of a 2' Ome oligonucleotide complementary to bases 29–45 of *Ascaris* U2 snRNA. Lanes designated T_0 are nuclease digestion controls (see text). Following incubation, 10 μ L splicing reactions were diluted to 30 μ L with splicing mix minus PEG (Hannon et al., 1990) plus 2 mM CaCl_2 prior to digestion on ice with 300 U micrococcal nuclease. Following addition of EGTA to 4 mM the samples were deproteinized and fractionated on 8% denaturing polyacrylamide gels prior to autoradiography. M: DNA markers of known size.

(-6), and (3) between the AG of the 3' splice site (-1). Each labeled RNA was then incubated in *Ascaris* extract under splicing conditions. Following incubation,

the reactions were quenched on ice and diluted with splicing mix lacking PEG and containing 2 mM CaCl_2 (Hannon et al., 1990) prior to addition of 300 U of micrococcal nuclease (Worthington). After a 10-min incubation on ice, digestion was terminated by addition of EGTA to 4 mM and the samples were deproteinized and fractionated on 8% denaturing polyacrylamide gels. We have used micrococcal nuclease because it is not sensitive to structure and can be readily inactivated. In principle, however, other nucleases could be employed. Regardless of the nuclease used, it is important to empirically determine the amount of nuclease required to give complete digestion of the labeled RNA in the presence of "carrier" RNAs contributed by the extract. In practice, to make this determination, we predilute extract in the presence of varying amounts of nuclease prior to addition of labeled RNAs. In our hands, this protocol precludes interaction of extract components with the labeled RNA and establishes a background from which to begin measuring specific protection. Against this background, we can readily detect nuclease-resistant fragments resulting from binding of factors (e.g., U1 snRNP) that rapidly associate with the RNA. For convenience, we refer to these reactions as T_0 in the figures. Although we have not explored the issue extensively, it seems intuitively preferable to perform digestions for as short a time as practicable to avoid potential dissociation.

With all three specifically labeled substrates, we observe a doublet of protected fragments of ~ 48 and ~ 38 nt in length. An additional cluster of shorter protected bands is seen only with RNAs labeled within the pyrimidine tract or 3' splice site (Fig. 2). The fact that different patterns of protection are evident when the label is located at different positions highlights the utility of using multiple labels.

Notably, the larger sets of bands are not evident if U2 snRNP is inactivated by a 2' Ome oligonucleotide complementary to the branch point recognition sequence; the smaller fragments are not effected by the oligonucleotide (Fig. 2, lanes 3, 6, and 9). Accordingly the larger fragments depend upon U2 snRNP binding to the substrate; the relevance of these observations is discussed elsewhere (C.M. Romfo, P.A. Maroney, T.W. Nilsen, in prep.). Of necessity, the observed protected fragments contain the specific label. However, the label could be located at any position within a given fragment. Therefore, to determine the sites of protection, it is necessary to map the boundaries of the fragments. This is easily done by excising the fragments from preparative gels and digesting them using the site-specific RNase H method. Such a mapping exercise is shown in Figure 3. Here, we excised the largest set of protected fragments (~ 48 nt) from reactions with branch point-labeled substrate (Fig. 2, lane 2, fragments A) and used site-specific RNase H digestion to cleave them (Fig. 3). Digestion in the presence of the oligo-

FIGURE 3. Mapping of protected fragments using site-specific RNase H digestion. Fragment set A from the gel shown in Figure 2, lane 2, was excised, purified, and aliquots were either rerun (lane –) or digested with RNase H in the presence of the 2' Ome/DNA oligonucleotides (as indicated) used to generate site-specifically labeled pre-mRNAs (see the legend to Fig. 2). For small-scale RNase H digestions, gel purified RNAs were incubated with 50 ng of the chimeric oligonucleotide and 5 U of enzyme. Labeled fragments visualized after digestion are indicated schematically in red. The apparent reduction in size heterogeneity of the purified fragments results from a combination of excision from the preparative gel and reduced exposure. The deduced region of protection on the pre-mRNA is indicated schematically in blue. A: alkaline ladder (Valadkhan & Manley, 2000); M: DNA markers of known size (indicated).

nucleotide used to generate the labeled pre-mRNA (BP) yielded a labeled fragment of 27 nt (Fig. 3, BP). Because RNase H cleavage leaves 5' phosphate and 3' hydroxyl termini, the labeled 27-mer is 3', indicating that the protected fragment extends from –46 to +2 relative to the 3' splice site junction. Independent digestion of the 48-mer with the 2' Ome/DNA oligonucleotides used to generate the Py- or AG-labeled RNAs yielded labeled fragments of 40 and 45 nt, respectively, confirming this interpretation.

DISCUSSION

We have described a simple and rapid method for determining sites and extents of protein–RNA interactions. To date, we have used this method to measure snRNP–pre-mRNA interactions (Maroney et al., 2000) and the binding of purified proteins to RNA (Wu et al., 1999). This reverse footprinting technique should be applicable to a wide variety of questions and it provides significant practical advantages over

widely used methods such as gel-mobility shift, toe-printing, and footprinting assays. Traditional footprinting requires the use of end-labeled molecules, a condition that imposes significant size constraints on the RNAs that can be examined. Similarly, gel-mobility shifts can be affected by the size of the labeled RNA, and subtle shifts can be obscured by a background of unshifted probe. The nuclease-digestion method eliminates unbound probe and, because it is rapid, minimizes problems in detection resulting from dissociation (a real concern in gel-shift techniques). Dissociation is also a potential problem in toe-printing because of the extensive manipulations involved. Finally, using site-specific labeling, it is possible to “survey” a molecule of interest for specific sites of RNA–protein interaction by “sprinkling” labels throughout the molecule and measuring protection of each site independently.

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