fraction of the lifetime. Original computations of McCamon and Karplus employing the methods of molecular dynamics predict restricted tyrosine motions of this amplitude with a quasi-harmonic character completed in picosecond times. A more detailed explicit analysis of the resulting depolarization of the fluorescence has been carried out by Levy & Szabo (1982), who show that stable depolarization values would be reached in about 2 ps. The present observations register the expected short-time depolarization but cannot unequivocally exclude energy transfer among the tyrosines as a partial or unique contribution to this effect. It is of interest to note that if the fast depolarization is assumed to be wholly due to the rotations of the tyrosines, the ratio $FLA(1)/A(0)$ observed, 0.78, virtually coincides with the average value resulting from the ultrafast motion of the four tyrosines calculated by Levi & Szabo (1982) for one of their models [see second column of Table I of Levi & Szabo (1982)]. A compact protein molecule with a single tyrosine will be required to test the predictions of molecular dynamics. This appears as a worthwhile undertaking because the analysis of the fast motions of proteins by this method offers considerable promise, and an experimental verification of some of its basic predictions would enlarge its scope and increase confidence in its application.

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A New Method To Characterize the Translation Initiation Sites of Messenger Ribonucleic Acids†

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ABSTRACT: RNA can be radiolabeled in vitro with ^{125}I in uridine and cytidine residues by initial mercuration of these pyrimidine residues by the method of Dale et al. [Dale, R. M. K., Martin, E., Livingston, D. C., & Ward, D. C. (1975) *Biochemistry 14*, 2447–2457] and subsequent electrophilic displacement of the mercury with $^{125}I^+$ generated by the method of Commerford [Commerford, S. L. (1971) *Biochemistry 10*, 1993–2000]. These two reactions can be manipulated to produce intact high specific activity ^{125}I-labeled mRNA containing approximately equal specific activity in their 5-[^{125}I]iodouridine and 5-[^{125}I]iodocytidine residues. In vitro radiolabeling of satellite tobacco necrosis virus RNA (STNV RNA) by this procedure yields a ^{125}I-labeled mRNA that is biologically active in ribosome protection analyses in that one obtains the correct translation initiation fragments of the mRNA. Exhaustive digestion of a specific ^{125}I-labeled 32 nucleotide long initiation fragment of STNV RNA using four separate nucleotide-specific digestion (hydrolysis) reactions yields four different populations of ^{125}I-labeled digestion products that can be resolved by two-dimenstional fingerprint procedures. Characterization of all these ^{125}I-labeled digestion products followed by overlap nucleotide sequence comparisons of these specific digestion products allows a nucleotide sequence determination of the original 32 nucleotide long translation initiation fragment of this mRNA. This suggests that this overall in vitro procedure can be used to determine the nucleotide sequence of the translation initiation site(s) of any mRNA.

Ribosome protection analyses of the nucleotide sequence of the translation initiation site(s) of mRNAs (Steitz, 1969) require high specific activity mRNAs with uniformly distributed radiolabel(s). Messenger RNAs derived from single cell or tissue culture systems are ideally suited for such analyses for one can usually prepare high specific activity uniformly radiolabeled mRNAs from such systems by in vivo labeling with $^{32}P_i$ or combinations of other nucleic acid precursors. In contrast, many multicelled or whole organism systems have slow growth rates or endogenous pools of nucleic acid precursors that restrict in vivo radiolabeling procedures. The resultant lack of uniformly radiolabeled, high specific activity

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mRNAs from such multicelled systems has limited the use of ribosome protection analysis to interpret features of mRNAs that influence the initiation of translation.

In vitro radiolabeling of isolated mRNAs offers a solution to this problem of preparation of radiolabeled mRNAs for ribosome protection analyses. However, in vitro radiolabeling procedures either tend to be nucleotide specific [e.g., Commerford's (1971) cytidine-specific radioiodination procedure] or fail to yield the greater than 1×10^6 dpm/μg of mRNA required for efficient ribosome protection analyses. Thus, previously reported attempts to use in vitro radiolabeled mRNA to analyze nucleotide sequences of translation initiation sites of mRNAs have failed (Legon et al., 1976).

This paper reports a new in vitro radiolabeling procedure for RNAs that readily yields intact high specific activity radioiodinated mRNAs containing greater than 1×10^6 dpm/μg of RNA distributed approximately equally between 5-[^{125}I]-iodouridine and 5-[^{125}I]iodocytidine residues. This paper further reports that radioiodinated mRNAs prepared by this in vitro method function correctly in ribosome protection analyses so as to facilitate the use of ribosome protection analysis to characterize the nucleotide sequences of the translation initiation site(s) of any mRNA.

Experimental Procedures

Materials. STNV[1] RNA was prepared from germinating mung beans infected with the B strain of tobacco necrosis virus and the SV1 strain of STNV (Clark & Klein, 1974). The 5′-terminal region of the resultant STNV RNA was identical with that previously characterized (Leung et al., 1979; Ysebaert et al., 1980) except that the 22nd nucleotide from the 5′ terminus of the STNV RNA used here had mutated or converted (Donis-Keller et al., 1981) from a U to a C residue. Cell-free 30000*g* supernatant (S-30) extracts of wheat germ were prepared by the method of Roberts & Patterson (1973) and then stored (without previous incubation) at −80 °C until used. Sources of specific reagents were as follows: carrier free Na^{125}I, Amersham Corp.; ^{203}Hg(OAc)$_2$, New England Nuclear Co.; CMCT and RNase U$_2$, Calbiochem Corp.; RNase A and spleen phosphodiesterase, Worthington Corp.; RNase T$_1$, P-L Biochemicals; proteinase K, Boehringer Mannheim Biochemicals.

Preparation of STNV [^{125}I]RNAs. Cytidine-specific in vitro radioiodination of STNV RNA was patterned after that of Commerford (1971) as described by Browning et al. (1980) and yielded STNV [C-^{125}I]RNA containing >98% of its ^{125}I label at 5-[^{125}I]iodocytidine. Uridine- and cytidine-specific in vitro radioiodination of STNV RNA featured a two-step reaction combining the mercuration procedure of Dale et al. (1975) and the radioiodination procedure of Commerford (1971). Specifically, 100 μg of STNV RNA was initially mercurated in 833 μL of 1.3 mM Hg(OAc)$_2$–8 mM NaOAc, pH 6.0, at 65 °C for 1 h. The mercuration reaction was terminated by the addition of 83 μL of cold (0 °C) quench buffer (2 M NaCl, 20 mM Na$_2$EDTA, 50 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5), and the STNV HgRNA was then recovered by addition of 1.8 mL of cold ethanol) and 0.9 mL of ether, followed by storage overnight at −20 °C and subsequent centrifugation (30 min, 16000*g*). The resultant STNV HgRNA was washed to remove residual Hg^{2+} by dissolving the STNV HgRNA pellet in 100 μL of 0.1 M NaOAc, pH 6.0, precipitation by addition of 200 μL of ethanol, storage for >6 h at −20 °C, and centrifugation as before. The STNV HgRNA was then dried (lyophilization) and, if necessary, stored at −20 °C, prior to further use. Use of ^{203}Hg(OAc)$_2$ in the above procedure establishes that this STNV HgRNA contains mercury in approximately 5% of its total nucleotides.

Dissolved STNV HgRNA (100 μg/50–100 μL of H$_2$O) was radioiodinated by the method described for the preparation of STNV [C-^{125}I]RNA (Browning et al., 1980) with two exceptions. First, the Li^+ salt of STNV HgRNA is quite insoluble and must be heated at 60 °C for 5 min to dissolve the preparation prior to radioiodination. Second, unless specified otherwise, the number of moles of $^{125}I^-$ used in the radioiodination was set at 4.5% of the total nucleotides in the RNA. STNV [U,C-^{125}I]RNA prepared by this procedure has a specific activity of $>2 \times 10^7$ dpm/μg and contains approximately equal specific activity ^{125}I label in both 5-[^{125}I]-iodoUp and 5-[^{125}I]iodoCp.

Formation, Isolation, and Recovery of ^{125}I-Labeled Initiation Fragments of STNV [U,C-^{125}I]RNA. 80S translation initiation complexes were prepared from wheat germ S-30 extracts with 100 μg of STNV [U,C-^{125}I]RNA in 1-mL reactions according to the ribosome protection procedure of Browning et al. (1980). These initiation complexes were then treated with RNase T$_1$ (300 units/mL) for 15 min at 25 °C, and the resulting ^{125}I-labeled materials associated with the ribosomes were then pelleted through an 8-mL cushion of cold (4 °C) 15% (w/v) sucrose, 0.1 M KCl, 3.0 mM Mg(OAc)$_2$, and 20 mM K^+-Hepes salt, pH 7.6, by centrifugation (150000*g*, 90 min, 4 °C). The resultant ^{125}I-labeled ribosome protected fragments of STNV [U,C-^{125}I]RNA were finally recovered by electrophoretic resolution on 20% polyacrylamide–7 M urea gels and subsequent elution by the method of Browning et al. (1980) with the exception that no carrier RNA was added. All subsequent characterizations utilized the now U,C-^{125}I-labeled 32 nucleotide long translation initiation fragment of STNV RNA (hereafter called [U,C-^{125}I]32mer) originally characterized by Browning et al. (1980).

Base-Specific Exhaustive Digestion (Hydrolysis) of U,C-^{125}I-Labeled RNA Fragments. Pyrimidine-specific exhaustive digestion of [U,C-^{125}I]32mer was obtained in 1-h, 37 °C incubations of reactions containing 10 μL of sterile water, (50–100) $\times 10^3$ cpm of [U,C-^{125}I]32mer, and 0.5 μg of RNase A. These reactions were then dried (lyophilization), freed of residual oligo- and mononucleotide cyclic 2′,3′-phosphates by incubation in 10 μL of 50 mM HCl for 30 min at 30 °C, and then dried by lyophilization prior to two-dimensional fingerprint analysis.

Cytidylate-specific exhaustive digestion of [U,C-^{125}I]32mer was obtained in a two-step process. First, (50–100) $\times 10^3$ cpm of [U,C-^{125}I]32mer was dissolved in 10 μL of 1 mM Na$_2$EDTA–10 mM Tris-HCl, pH 8.9, containing 1 mg of CMCT and incubated for 12 h at 50 °C to derivatize all the U and G residues in the [U,C-^{125}I]32mer with CME-amidino blocking groups. The derivatized [U,C-^{125}]32mer was recovered by addition of 40 μL of 0.2 M NaOAc, pH 4.5, and

[1] Abbreviations: 5-[^{125}I]iodoUp, 5-[^{125}I]iodouridylate; 5-[^{125}I]iodoCp, 5-[^{125}I]iodocytidylate; [C-^{125}I]RNA, an RNA containing ^{125}I label in 5-[^{125}I]iodoCp as a result of radioiodination by the procedure of Commerford (1971); [U,C-^{125}I]RNA, an RNA containing ^{125}I label in 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp as a result of the procedure described in this paper; STNV, satellite tobacco necrosis virus; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; CME-amidino adduct, a 1-cyclohexyl-3-(2-morpholinoethyl)amidino nucleotide adduct as a result of reaction with CMCT; 32mer, a 32 nucleotide long initiation fragment that arises from nucleotides at positions 12–44 from the 5′ terminus of STNV RNA; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate.

150 μL of ethanol, storage at −20 °C overnight, and centrifugation (16000*g*, 30 min). The derivatized [U,C-^{125}I]32mer was then dissolved in 10 μL of H_2O containing 0.5 μg of RNase A and incubated at 37 °C for 1 h prior to inactivation of the RNase A by initial addition of 1 μL of a freshly prepared 5 mg/mL proteinase K solution, incubation for 2 h at 37 °C, further addition of another 1 μL of fresh 5 mg/mL proteinase K solution, and further incubation for >2 h at 37 °C. The CME-amidino blocking groups on U and G residues of the digestion products were then removed by addition of 1.2 μL of concentrated NH_4OH and incubation for 16 h at 25 °C, prior to final lyophilization to dryness and two-dimensional fingerprint analysis.

Adenylate-specific exhaustive digestion of [U,C-^{125}I]32mer was obtained in a two-step process similar to the cytidine-specific exhaustive digestions. First (50–100) × 10^3 cpm of [U,C-^{125}I]32mer was reacted with CMCT and recovered as described above. The derivatized [U,C-^{125}I]32mer was then incubated for 2 h at 37 °C in 10 μL of freshly prepared 7 M urea, 1 mM Na_2EDTA, and 20 mM sodium citrate, pH 3.5, containing 3.3 units of RNase U_2. The resulting oligo- and mononucleotides were then freed of urea by adsorption onto 2–3 mg of DEAE-cellulose and three successive washes by suspension in, and centrifugation (microfuge) from, 0.1-mL aliquots of 95% ethanol. The ^{125}I-labeled materials were then washed from the DEAE-cellulose by suspension in 0.2 mL of 30% (v/v) TEAB, pH 9.0, centrifugation (microfuge), and aspiration of the soluble ^{125}I-labeled materials. This preparation was freed of TEAB by lyophilization to dryness and then freed of amidino blocking groups as described above, prior to final lyophilization to dryness and two-dimensional fingerprint analysis.

Guanylate-specific exhaustive digestion was obtained by a successive mixing of (50–100) × 10^3 cpm of [U,C-^{125}I]32mer with 5 μg of unlabeled STNV RNA, lyophilization to dryness, resuspension in 10 μL of water containing 0.07 unit of RNase T_1, incubation for 1 h at 37 °C, and final lyophilization to dryness prior to two-dimensional fingerprint analysis.

Two-Dimensional Electrophoresis—Homochromatographic Fingerprint Resolution of U,C-^{125}I-Labeled Oligo- and Mononucleotides. Each base-specific digestion reaction mixture was separately dissolved in 2.5 μL of pH 2.5 electrophoresis buffer (2 mM Na_2EDTA–7 M urea, titrated to pH 2.5 with 90% formic acid), spotted onto 3 × 50 cm cellulose–acetate strips (Schleicher & Schuell Co.) and resolved electrophoretically (3000 V) until an added xylene cyanole FF dye migrated 10 cm toward the (+) pole. The ^{125}I-labeled materials on the cellulose–acetate strip between the origin and 20 cm toward the (+) pole were then transferred to a 20 cm × 20 cm DEAE-cellulose thin-layer plate (Polygram Cel 300 DEAE/HR-2/15, Brinkmann Instrument Co.) by the method of Southern (1974) with the modification that the transfer of products rich in protonated adenosine was enhanced by initial titration of the cellulose–acetate strip containing ^{125}I-labeled materials to neutral pH by 5-min exposure to NH_3 vapors and subsequent vacuum removal of residual NH_3. The transferred ^{125}I-labeled compounds were then further resolved in the second dimension by homochromatography (Silberklang et al., 1977) at 60 °C with a 25 mM KOH homochromatographic mixture and finally detected by radioautography.

Characterization of ^{125}I-Labeled Compounds Detected on Two-Dimensional Fingerprints. All the ^{125}I-labeled compounds detected on two-dimensional fingerprints were subjected to a sufficient number of the following four analysis procedures to establish the correct nucleotide composition and sequence of each ^{125}I-labeled compound. Each of these assays employed at least 1000 cpm, and where possible up to 20 000 cpm, of ^{125}I-labeled material and featured sterilized solutions to avoid nuclease contaminations.

First, the size and relative proportion of ^{125}I label in each radioiodinated compound were determined. This approach featured initial size estimation from the degree of homochromatographic resoltuion on the fingerprint. This was followed by a successive scraping of the DEAE-cellulose from the separate zones of ^{125}I labeling on the fingerprints, detection of the cpm of ^{125}I label present in each scraped sample, and removal of contaminating urea from the separate powder samples by three successive washes by suspension in, and centrifugation (microfuge) from, 0.1-mL aliquots of 95% ethanol. The separate ^{125}I-labeled materials were then recovered from the DEAE-cellulose by suspension in 0.2 mL of 30% v/v TEAB, pH 9.0, centrifugation (microfuge), aspiration of the soluble ^{125}I-labeled materials, and lyophilization to dryness. The size of each separate ^{125}I-labeled compound was confirmed by dissolution of each extracted sample in 20 μL of H_2O, homochromatographic resolution of 1–5-μL aliquots against ^{125}I-labeled standards (obtained from STNV [U,C-^{125}I]RNA by exhaustive digestion with RNase A), and final radioautography.

Second, the relative proportion of 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp present in each ^{125}I-labeled compound extracted from the fingerprint (as above) was determined by exhaustive digestion with RNase T_2 and subsequent analyses. These assays employed 2–3-h, 37 °C incubations of separate 5-μL aliquots of the dissolved compounds with 5 μL of 0.1 M ammonium acetate buffer, pH 5.3, containing 0.02 unit of RNase T_2. This was followed by one-dimensional, 3000-V, electrophoretic resolution of the hydrolyzed samples (and 2000 cpm of 5-[^{125}]iodoUp and 5-[^{125}I]iodoCp standards) on Whatman No. 3 MM paper in pH 2.5 electrophoresis buffer until an added xylene cyanole FF dye marker migrated 10 cm. ^{125}I-Labeled materials were detected and quantitated by radioautography and γ counting of cut-out paper zones.

Third, where necessary, dissolved 5–10-μL aliquots of the individual ^{125}I-labeled compounds were further digested exhaustively with one or more of the base-specific digestions discussed above, followed by one- or two-dimensional analyses of the ^{125}I-labeled secondary digestion products. Exhaustive digestion procedures were as described previously except that lower levels of ^{125}I label were often employed and the ^{125}I-labeled materials present after derivatization with CMCT were recovered by adsorption onto DEAE-cellulose and extraction with TEAB, pH 9.0, as described for the removal of urea during the adenylate-specific exhaustive digestion. Often a full characterization of the products of base-specific secondary exhaustive digestions could be obtained from knowledge of the base-specific digestion used to generate the original compound, the specificity of the secondary digestion employed, and one-dimensional 25 mM KOH homochromatographic resolution (Silberlang et al., 1977) against ^{125}I-labeled size standards. Sometimes a full characterization of the products of a base-specific exhaustive secondary digestion required identification and quantitation of the amounts of 5-[^{125}I]-iodoUp and 5-[^{125}I]iodoCp present in the various secondary digestion products detected on one- or two-dimensional analyses. In these cases, the ^{125}I-labeled compounds in question were extracted from the one- or two-dimensional system and then further exhaustively digested with RNase T_2 and analyzed by one-dimensional electrophoresis at pH 2.5 as described above. Occasionally, a full characterization of the products

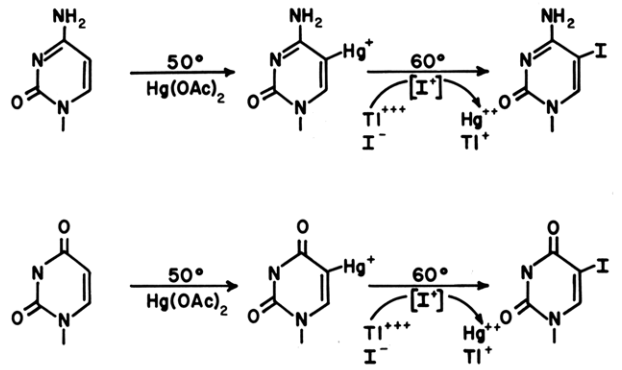

FIGURE 1: Two-step scheme to iodinate pyrimidines.

of a base-specific secondary digestion required use of an additional base-specific tertiary digestion of the base-specific secondary digestion products detected by one- or two-dimensional analyses. In these case, base-specific tertiary digestions were carried out identically with the base-specific secondary digestion described above. Lastly, as noted above, most analyses of base-specific exhaustive digestions utilized one-dimensional homochromatographic resolution of the secondary or tertiary digestion products. A few specific (and large) oligonucleotides can not be fully characterized by use of the base-specific exhaustive digestion procedures and the total (RNase T_2 dependent) digestion procedures (described above) in combination with one-dimensional resolutions of the ^{125}I-labeled products. In these cases, the ^{125}I-labeled products of base-specific secondary digestions were resolved by the two-dimensional fingerprint procedure described above, followed by extraction of the individual ^{125}I-labeled compounds from the fingerprint and further base-specific analyses or analyses of 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp content, as described above, or determinations of "*M* values" as described below.

Fourth, the character of all compounds generated by pyrimidine-specific exhaustive primary, secondary, or tertiary digestions was established by the use of the *M* values method of Brownlee (1972). Specifically, (1–10) × 10^3 cpm of a specific ^{125}I-labeled compound was lyophilized to dryness, taken up in 10 μL of 2 mM Na_2EDTA–0.1 M potassium phosphate, pH 6.5, containing 0.05 unit of spleen phosphodiesterase, and incubated at 37 °C. Aliquots (2 μL) were removed from each reaction after 0, 1, 5, 10, and 30 min of incubation, boiled for 1 min, stored at −80 °C, and then combined before spotting onto DEAE-cellulose paper prewetted with pH 1.9 electrophoresis buffer (3.5% formic acid–8.7% acetic acid), and electrophoretic resolution (3000 V) was until an added xylene cyanole FF dye marker migrated 5 mm toward the (+) pole. ^{125}I-Labeled compounds were then detected by radioautography and characterized by *M* values.

Results

Specificity of ^{125}I-Labeling Reactions. Dale et al. (1975) report that incubation of RNA with Hg^{2+} salts under conditions that favor disruption of secondary structure (50 °C, low salts) results in the derivatization of uridine and cytidine residues to form 5-mercuriuridine and 5-mercuricytidine in the RNA. I^+ is a stronger electrophile than the mercury of 5-mercuripyrimidines. It follows that $^{125}I^+$ should displace the mercury from 5-mercuripyrimidine residues in RNAs to yield [U,C-^{125}I]RNAs containing both 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp. Commerford's (1971) in vitro radioiodination procedure generates $^{125}I^+$ by Tl^{3+}-dependent oxidation of $^{125}I^-$. Use of $^{125}I^-$ and RNAs containing 5-mercuriuridine and 5-mercuricytidine in Commerford's (1971) iodination procedure

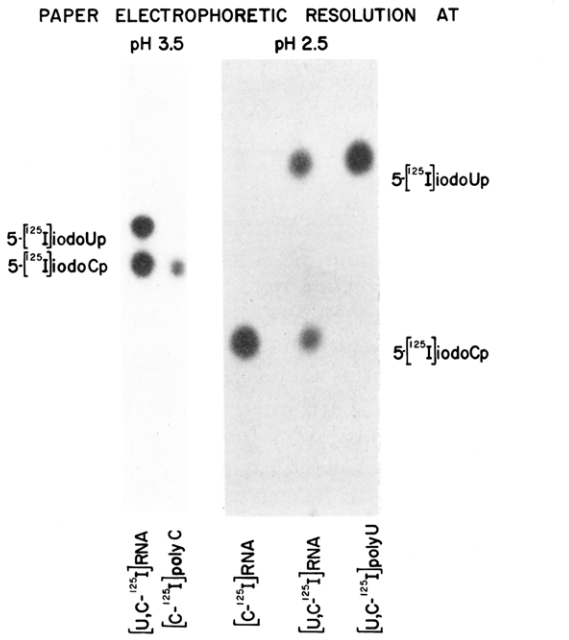

FIGURE 2: Radioautograms of paper electrophoretic resolution of 5-[^{125}I]iodouridylate and 5-[^{125}I]iodocytidylate derived from [C-^{125}I]RNAs and [U,C-^{125}I]RNAs by exhaustive digestion with RNase T_2.

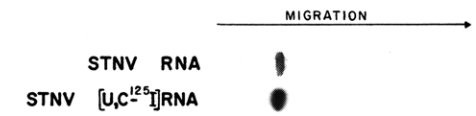

FIGURE 3: Gel electrophoretic resolution of STNV RNA and STNV [U,C-^{125}I]RNA on 4% polyacrylamide gel by the method of Leung et al. (1979). STNV RNA is visualized by staining with Stains-All (Miles Labs). STNV [U,C-^{125}I]RNA is detected by radioautography.

should therefore yield [U,C-^{125}I]RNA containing both 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp (Figure 1).

The scheme and specificity of figure 1 are correct for [U,C-^{125}I]RNA prepared by initial mercuration (Dale et al., 1975), and subsequent radioiodination (Commerford, 1971) yields 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp as the only radioactive products when digested to constituent nucleotides and analyzed by paper electrophoretic resolution (Figure 2). As noted by Robertson et al. (1973), 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp migrate with similar R_fs during electrophoresis at pH 3.5. 5-[^{125}I]Iodocytidine must therefore have a pK_a lower than that of cytidine, and one must employ lower pHs, for example, pH 2.5, to protonate 5-[^{125}I]iodoCp significantly and generate differential electrophoretic migration of 5-[^{125}]iodoCp from 5-[^{125}I]iodoUp. It follows that electrophoretic resolutions of 5-[^{125}I]iodoCp and 5-[^{125}I]iodoUp containing oligonucleotides will also benefit from electrophoretic resolution at a low pH such as pH 2.5.

Three features of the reactions of Figure 1 suggest potential concerns about the biological usefulness of [U,C-^{125}I]RNAs. First, the reactions feature conditions that can potentially favor cleavages of RNAs. Yet, as seen in Figure 3, the relatively large RNA, STNV RNA, yields intact STNV [U,C-^{125}I]RNA when treated by the reaction scheme of Figure 1. Cleavage of RNA is, therefore, not a concern if one starts with a nuclease-free RNA. Second, [U,C-^{125}I]RNAs can contain 5-mercuripyrimidines due to incomplete displacement of the mercury. Such 5-mercuripyrimidines can react with SH groups on enzymes to inactivate the enzymes or alter the ^{125}I data obtained. Third, as noted earlier, 5-[^{125}I]iodoCp has a pK_a, and therefore an electrophoretic mobility, different from

Table I: Effect of Varying Levels of $^{125}I^-$ upon the Formation of 5-[^{125}I] Iodopyrimidine Nucleotides in STNV [U,C-^{125}I] RNA[a]

pmol of $^{125}I^-$ / pmol of HgRNA	cpm in ^{125}I-labeled nucleotides/μg of STNV RNA: 5-[^{125}I]-iodoCp	5-[^{125}I]-iodoUp	5′-[^{125}I]iodoCp / 5′-[^{125}I]iodoUp
1/10	1500	2800	0.54
1/2	3700	3200	1.16
1/1	6350	3650	1.74
2/1	9600	4450	2.16

[a] Each assay used STNV HgRNA containing 75 pmol of Hg/μg of RNA, i.e., approximately 5% mercuration of nucleotides. Yields of individual 5-[^{125}I] iodopyrimidine nucleotides were determined after exhaustive digestion of resultant STNV [U,C-^{125}I]-RNAs with RNase T_2 and subsequent paper electrophoretic resolution at pH 2.5.

cytidylate. Base-specific digestion of [U,C-^{125}I]RNAs carrying a high percentage of 5-[^{125}I]iodopyrimidines can, therefore, yield electrophoretic heterogeneity with ^{125}I-labeled oligonucleotide molecules that arise from the same original sequence of the [U,C-^{125}I]RNA (e.g., 5-[^{125}I]iodoUpCpGp vs. 5-[^{125}I]iodoUp[^{125}I]iodoCpGp). Such electrophoretic heterogeneity complicates characterizations required in practical uses of [U,C-^{125}I]RNAs.

We reasoned that one can minimize these concerns about residual mercury and electrophoretic heterogeneity in specific degradative products by using [U,C-^{125}I]RNAs that contain the minimum derivatization with Hg and ^{125}I required to obtain the ^{125}I specific activity desired. However, such incomplete reactions allow the specificities of the individual reactions to influence the resultant proportions of 5-[^{125}I]-iodopyrmidines produced. Dale et al. (1975) and Bergstrom & Ruth (1977) report that the mercuration of pyrimidines favors the mercuration of uridine residues over cytidine residues. Our studies with $^{203}Hg(OAc)_2$ confirm that at 65 °C one obtains a 10-fold greater rate of mercuration of uridine residues compared with that of cytidine residues in RNAs. In contrast to this biased mercuration reaction, Commerford's (1971) radioiodination procedure used to displace the mercury from 5-mercuripyrimidines also yields a largely cytidine-specific radioiodination of RNAs (Browning et al., 1980). Thus one can employ a limited low level of Hg^{2+} in the mercuration reaction and vary the quantity of $^{125}I^-$ in the radioiodination reaction to obtain various proportions of ^{125}I label in 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp in [U,C-^{125}I]RNAs (Table I). Most importantly, these data show that one can employ a limited mercuration of pyrimidines and then manipulate the level of $^{125}I^-$ in the radioiodination reaction to obtain approximately equal specific activity 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp in the final [U,C-^{125}I]RNA. Such equal specific activities simplify stoichiometric considerations in the characterization of [U,C-^{125}I]iodooligoribonucleotides derived from [U,C-^{125}I]RNAs.

Functionality of a [U,C-^{125}I]mRNA in Ribosome Protection Assays. Initial proof of the potentials of [U,C-^{125}I]mRNAs in ribosome protection assays requires proof that a [U,C-^{125}I]mRNA will yield correct initiation fragments. Accordingly, we chose to test STNV [U,C-^{125}I]RNA in a ribosome protection proocedure known to yield a specific set of well-characterized C-^{125}I-labeled initiation fragments from the monocistronic mRNA, STNV [C-^{125}I]RNA (Browning et al., 1980). As seen in Figure 4, STNV [U,C-^{125}I]RNA yields the same ^{125}I-labeled initiation fragments detected from STNV [C-^{125}I]RNA when both ^{125}I-labeled mRNAs are protected

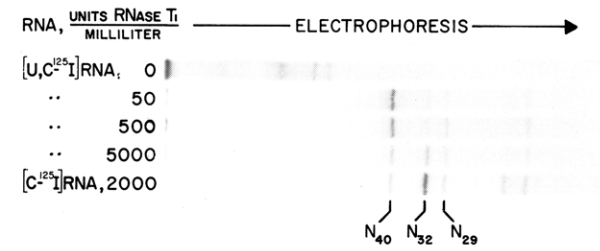

FIGURE 4: Radioautogram of gel electrophoretic resolution of ^{125}I-labeled initiation fragments of STNV [U,C-^{125}I]RNA and STNV [C-^{125}I]RNA protected from increasing concentrations of RNase T_1 by wheat germ 80S translation–initiation complexes. N_{40}, N_{32}, and N_{29} indicate the location of specific size standards, all resolved in parallel tracks on the gel.

from the action of RNase T_1 by wheat germ ribosomes. The extra ^{125}I label and the limited residual mercury present in STNV [U,C-^{125}I]RNA therefore do not affect the biological function of [U,C-^{125}I]mRNAs in ribosome protection procedures.

Final proof of the potentials of [U,C-^{125}I]mRNAs in ribosome protection assays requires proof that one can use base-specific digestions of a specific U,C-^{125}I-labeled initiation fragment, and subsquent overlap sequence analyses of the products from these base-specific digestions, to characterize the complete nucleotide sequence of the original [U,C-^{125}I]-initiation fragment. We chose to use the 32 nucleotide long U,C-^{125}I-labeled initiation fragment of STNV [U,C-^{125}I]RNA (Figure 4), hereafter referred to as [U,C-^{125}I]32mer, to test this point.

RNase A hydrolyzes RNAs 3′ adjacent to 5-iodoCp (Robertson et al., 1973). We therefore reasoned that RNase A dependent exhaustive digestion of [U,C-^{125}I]32mer would yield a series of radioiodinated products, each containing a single and 3′-terminal 5-[^{125}I]iodopyrimidine nucleotide. This reasoning is correct for RNase A dependent exhaustive digestion of [U,C-^{125}I]32mer containing equal specific activity 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp, followed by two-dimensional fingerprint resolution of the products, yields the radioiodinated products of Figure 5. Quantitative analysis of the dpm of ^{125}I label present in each radioiodinated compound reveals that RNase A produces approximately 4 molecules of U(Up), 2 molecules of AAC, and 1 molecule of the other products per molelcule of [U,C-^{125}I]32mer. This quantitation accounts for 30 of the 32 nucleotides in [U,C-^{125}I]32mer, and dictates that the 3′ terminus of [U,C-^{125}I]32mer contains two consecutive purine nucleotides, one of which must be the 3′ terminal guanylate generated by use of RNAse T_1 in the ribosome protection procedure.

As noted above, RNase A dependent exhaustive digestion of [U,C-^{125}I]32mer, and other U,C-^{125}I-labeled fragments, yields a uniform distribution of ^{125}I label in the radioiodinated products. However, the limitation of the ^{125}I label of U,C-^{125}I-labeled initiation fragments to 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp dictates that other base-specific exhaustive digestions of U,C-^{125}I-labeled initiation fragments can yield oligo- and mononucleotides lacking 5-[^{125}I]iodoUp and 5-[^{125}I]-iodoCp. Such unlabeled, and therefore undetectable, oligo- and mononucleotides complicate nucleotide sequence characterizations based upon overlap sequence comparisons. However, we reasoned that if one characterizes the ^{125}I-labeled products from a wide variety of base-specific, exhaustive digestions of a U,C-^{125}I-labeled initiation fragment, the wider variety of ^{125}I-labeled products obtained can overcome the limited distribution of ^{125}I label and allow a complete characterization of any U,C-^{125}I-labeled initiation fragment.

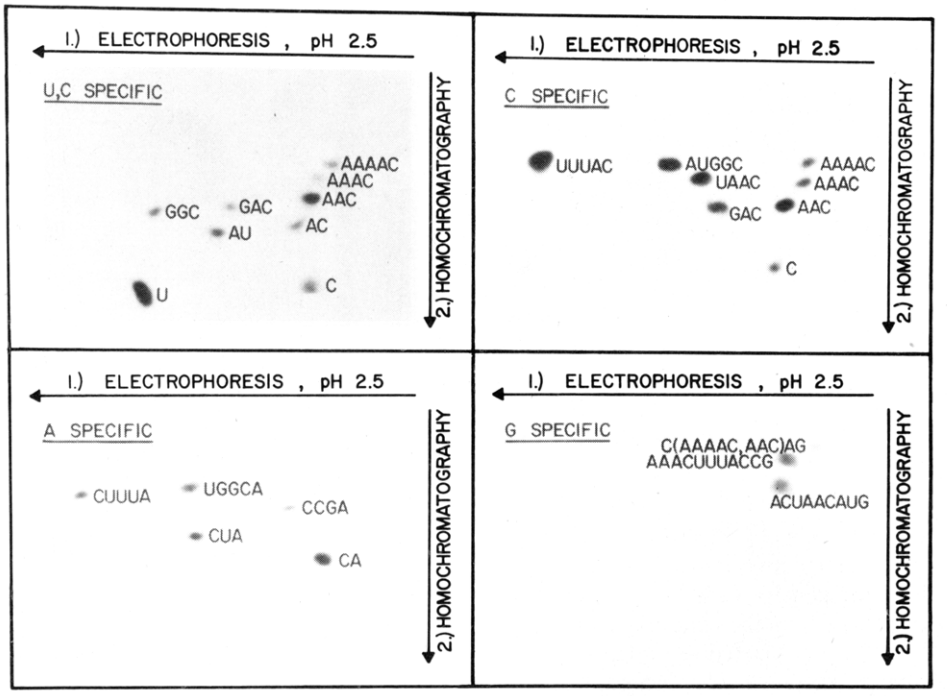

FIGURE 5: Radioautograms of two-dimensional fingerprint resolution of ^{125}I-labeled products released from [U,C-^{125}I]32mer by various base-specific exhaustive digestions.

Table II: Overlap Nucleotide Sequence Analysis of [U,C-^{125}I]32mer

^{125}I-labeled products from exhaustive digestions	
U,C specific	AAACUUUACCGACUAACAUGGCAAAACAAC
C specific	AAACUUUACCGACUAACAUGGCAAAACAAC
A specific	CUUUACCGACUA CAUGGCA CA CA
G specific	AAACUUUACCGACUAACAUG CAAAACAACAG
nucleotide sequence from overlap analysis	AAACUUUACCGACUAACAUGGCAAAACAACAG

Accordingly, we have used existing and newly developed procedures to obtain cleavage of U,C-^{125}I-labeled initiation fragments in a cytidine-specific, adenylate-specific, and guanylate-specific manner.

Cytidylate-specific cleavage of U,C-^{125}I-labeled initiation fragments can be obtained by use of the water-soluble carbodiimide CMCT. This reagent reacts with only uridine and guanosine residues of RNAs to yield bulky and cationic CME-amidino adduct derivatives (Gilham, 1962). The CME-amidino adduct of uridylate is not a substrate site for RNase A (Gilham, 1962; Dickson et al., 1979). Therefore, RNase A dependent exhaustive digestion of a [U,C-^{125}I]32mer that has been exhaustively derivatized with CMCT yields the C-specific radioiodinated products shown in Figure 5. Most importantly, each of these ^{125}I-labeled oligonucleotide products has a 3′ terminal 5-[^{125}I]iodoCp, so the process is cytidylate-specific and the 30 nucleotides detected in these ^{125}I-labeled products provide an additional set of nucleotide sequences for overlap comparisons and eventual characterization of the [U,C-^{125}I]32mer.

Adenylate-specific cleavage of U,C-^{125}I-labeled initiation fragments should be obtainable by use of RNase U_2 in urea environments (Donis-Keller et al., 1977). However, we find that use of RNase U_2 under these conditions does not yield a totally adenylate-specific cleavage of [U,C-^{125}I]32mer. For example, two-dimensional fingerprint analyses of the ^{125}I-labeled products released from [U,C-^{125}I]32mer by RNase U_2 in urea detect variable amounts of ACUUUA, ACCGA, AUGG, UGGCA, and CAACAG, indicating that, under these conditions, RNase U_2 has difficulty cleaving consecutive adenylate residues and that RNase U_2 does cleave after some guanylate residues. These partial digestion products are qualitatively useful in overlap comparisons to characterize the full nucleotide sequences of [U,C-^{125}I]32mer, but the limited and variable yield of these partial products precludes quantitative considerations associated with overlap comparisons of oligonucleotides. We therefore employed the guanosine- and uridine-specific derivatization properties of CMCT described above to achieve adenylate-specific cleavage of U,C-^{125}I-labeled initiation fragments. Specifically, exhaustive derivatization of [U,C-^{125}]32mer with CMCT will block all the guanosine and uridine residues. Such derivatization should eliminate RNase U_2 dependent cleavages after guanylate residues and allow use of the higher levels of RNase U_2 needed for RNase U_2 dependent digestion at all adenylate residues. Subsequent removal of the RNase U_2 and urea and final mild alkali dependent removal of the CME-amidino derivatization should allow two-dimensional fingerprint analysis of the adenylate-specific cleavage products. Figure 5 shows this line of experimentation to be correct for use of this CMCT-dependent and RNase U_2 dependent procedure on [U,C-^{125}I]32mer yields a specific set of ^{125}I-labeled oligonucleotides, each containing a 3′-terminal adenylate.

Guanylate-specific cleavage of U,C-^{125}I-labeled initiation fragments can be obtained through use of the well-known guanylate-specific properties of RNase T_1. As seen in Figure 5, RNase T_1 dependent digestion of [U,C-^{125}I]32mer yields three specific ^{125}I-labeled oligonucleotides. Summation of the 31 nucleotides accounted for in these sequences, and comparison with the 32 nucleotides known to be present in [U,C-^{125}I]32mer, establishes that the [U,C-^{125}I]32mer must have a single GG sequence somewhere in its overall sequence.

The characterization of each of the ^{125}I-labeled products obtained from the four different base-specific exhaustive di-

gestions of [U,C-^{125}I]32mer (Figure 5) provides sufficient data for an overlap nucleotide sequence analysis of the original [U,C-^{125}I]32mer (Table II). This analysis essentially involves ordering the three specific oligonucleotides released from [U,C-^{125}I]32mer by exhaustive digestion with RNase T_1. AUGGC (from cytidylate-specific cleavage of [U,C-^{125}I]-32mer) and UGGCA (from adenylate-specific cleavage of [U,C-^{125}I]32mer) dictate that the nanonucleotide ACUAACAUG must be followed by the G of the GG sequence established above, and this GG sequence must be followed by the oligonucleotide C(AAAAC,AAC)AG. Similarly, CCGA (from adenylate-specific cleavage of [U,C-^{125}I]32mer) dictates that the oligonucleotide AAACUUUACCG must be at the 5′ terminus of [U,C-^{125}I]32mer. Thus the nucleotide sequence of the 32 nucleotide long initiation fragment of STNV RNA, as derived by this procedure, is AAACUUUACCGACUAACAUGGC(AAAAC,AAC)AG.

The A-rich character of the sequence in parentheses near the 3′ terminus of [U,C-^{125}I]32mer precludes use of overlap nucleotide sequence analysis of the products from base-specific exhaustive digestions to establish the exact sequence in this region of [U,C-^{125}I]32mer (Table II). However, two additional approaches can be used to eliminate this sequence ambiguity. First, partial digestion of underivatized [U,C-^{125}I]32mer with RNase U_2 in urea yields CAACAG as one of the products. This must come from the six nucleotides at the 3′ terminus of [UC-^{125}I]32mer and therefore dictates that the correct sequence for [U,C-^{125}I]32mer is as written in Table II with the parentheses and comma removed. Second, any ambiguity on the 3′ side of an initiator codon can be resolved if one knows the N-terminal sequence of the protein whose translation begins in the initiation fragment. The N-terminal sequence of the initial product of STNV RNA translation is Met-Ala-Lys-Gln-Gln... (Leung et al., 1979). Genetic code assignments then establish the sequence of Table II, without the parentheses and comma, as the correct nucleotide sequence of [U,C-^{125}I]32mer. This characterization of the nucleotide sequence of the [U,C-^{125}I]32mer also agrees with previous characterizations of this region of STNV RNA (Leung et al., 1979; Browning et al., 1980).

Discussion

The data presented here establish that our in vitro uridine- and cytidine-specific radioiodination and associated ribosome protection procedure can yield the nucleotide sequence of the translation initiation region of a mRNA. It should be emphasized that although this paper characterizes a previously known nucleotide sequence, the methods presented here yield the nucleotide sequence of the translation initiation region of STNV RNA without reference to previous characterization data. These methods should therefore also yield the nucleotide sequence of the translation initiation site(s) of any mRNA.

This report also presents cytidylate- and adenylate-specific cleavage reactions for use with oligoribonucleotides and possibly larger RNA molecules. Use of these newly developed base-specific cleavage reactions on an U,C-^{125}I-labeled initiation fragment provides the wider variety of base-specific cleavages needed to overcome the limited distribution of ^{125}I label in U,C-^{125}I-labeled initiation fragments. The same CMCT-dependent total derivatizations of uridines and guanosines that facilitate the cytidylate- and adenylate-specific exhaustive digestions should also allow RNase A and RNase U_2 dependent *partial* digestion of oligonucleotides and possibly larger RNA molecules. Thus these cytidylate- and adenylate-specific reactions can potentially aid a wide variety of RNA sequence characterizations. Most importantly for the overall method reported here, these cytidylate- and adenylate-specific reactions function effectively and specifically with small quantities of small oligonucleotides. This allows use of a full range of base-specific secondary and tertiary digestions on isolated [U,C-^{125}I]iodooligonucleotides and serves as the basis for the characterization of the specific [U,C-^{125}I]iodooligonucleotides that allows the overlap nucleotide sequence analysis emphasized in this paper.

Two features of the mercuration step of the two-step radioiodination procedure reported here deserve further comment. First, as noted, the mercuration reaction conditions employed in these preparations yield HgRNA containing mercury in approximately 5% of its total nucleotides. The level of ^{125}I$^-$ used to produce a [U,C-^{125}I]RNA with approximately equal specific activity 5-[^{125}I]iodoUp and 5-[^{125}I]iodoCp (i.e., 4.5% of total nucleotides) displaces some of the mercury from HgRNA. However, the final [U,C-^{125}I]RNA still contains HgRNA (Hg on 3–4% of the total nucleotides). Interestingly, this low level of mercuripyrimidines in such [U,C-^{125}I]RNA does not restrict its use in ribosome protection assays (Figure 4) and studies with specific nucleases (Figure 5). It remains to be seen if such low levels of mercuripyrimidines in [U,C-^{125}I]RNAs restrict or inhibit other enzyme assays. Second, the mercuration step of the two-step radioiodination procedure reported here may also provide a probe for secondary structures in RNAs. Dale et al. (1975) use 37–50 °C to facilitate the mercuration of pyrimidine nucleotides and polynucleotides. Use of 50 °C in the mercuration step of the two-step radioiodination of STNV RNA described here results in a greatly reduced isolation of 5[^{125}I]iodoUp derived from [U,C-^{125}I]-32mer by exhaustive digestion with RNase A (i.e., products distributed as the U,C-specific digestion products of Figure 5). Three of the four uridylate residues that should yield 5-[^{125}I]iodoUp in this procedure are located at positions 17–19 from the 5′ terminus of STNV RNA in an H-bonded secondary structure of STNV RNA (Browning et al., 1980). We reasoned that such H bonding may restrict the mercuration step in our two-step radioiodination of STNV RNA. Accordingly, we used 65 °C in all subsequent mercuration steps of our two-step procedure. This higher temperature in the mercuration step always yields the expected levels of 5-[^{125}I]iodoUp, suggesting that some secondary structural considerations of STNV RNA influence the mercuration of STNV RNA at lower temperatures.

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Effects of Manganous Ion on the Phosphorus-31 Nuclear Magnetic Resonance Spectrum of Adenosine Triphosphate Bound to Nitrated G-Actin: Proximity of Divalent Metal Ion and Nucleotide Binding Sites†

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ABSTRACT: G-Actin has one high-affinity binding site for ATP and one high-affinity binding site for divalent metal ions such as Ca^{2+}, Mg^{2+}, or Mn^{2+}. ^{31}P NMR has been used to study the high-affinity ATP binding site of a relatively nonpolymerizable selectively nitrated derivative of G-actin. When paramagnetic manganous ion was added to nitrated G-actin, the line widths of the resonances for the α-, β-, and γ-phosphates of the bound ATP did not increase substantially. However, the areas of the resonances of all three phosphates decreased with increasing concentration of manganous ion. This decrease in area paralleled a decrease in tightly bound calcium displaced by the manganous ion. Manganese-induced polymerization of the nitrated G-actin was found to be a relatively minor process in these experiments. The ^{31}P NMR results are consistent with very slow exchange between the Ca^{2+}·ATP·nitrated G-actin complex and the Mn^{2+}·ATP·nitrated G-actin complex. Thus, the areas of the observed resonances, which represent the Ca^{2+}·ATP·nitrated G-actin complex, vary as a function of the population of this complex, but the line widths are not affected by exchange with the Mn·ATP·nitrated G-actin complex. The line widths of the ^{31}P NMR resonances of the bound ATP in the Mn·ATP·nitrated G-actin complex are too broad to be detected (greater than 400 Hz) due to the paramagnetic effect of the tightly bound manganous ion. This indicates that the high-affinity metal ion binding site on G-actin (occupied by manganous ion) must be less than 10 Å from the ATP binding site.

G-Actin is a globular protein of molecular weight 42 300 (Elzinga et al., 1973) that plays an essential role in the contractile event of muscle cells as well as in various motility processes of nonmuscle cells. G-Actin has three distinct classes of divalent metal ion binding sites. There is one high-affinity binding site primarily for divalent metal ions ($K_D \simeq 10^{-5}$ M)[1] which is required for protein stability (Bárány et al., 1962; Martonosi et al., 1964; Kasai et al., 1965; Frieden et al., 1980). A second class of five to seven lower affinity binding sites ($K_D \simeq 10^{-3}$ M) are implicated in the polymerization of G- to F-actin (Martonosi et al., 1964; Oosawa & Kasai, 1971). Finally, there are low-affinity sites ($K_D \simeq 10^{-2}$ M) which appear to be involved in the formation of paracrystals from F-actin filaments (Hanson, 1973; Strzelecka-Golaszewska et al., 1978). Various studies have shown that several different divalent cations can bind interchangeably to the same high-affinity metal ion binding site, particularly Ca^{2+}, Mg^{2+}, and Mn^{2+} (Drabikowski & Strzelecka-Golaszewska, 1963; Kasai & Oosawa, 1968).

G-Actin requires bond nucleotide, usually ATP, to maintain its structural integrity (Laki et al., 1950; Straub & Feuer, 1950). ATP binds in a 1:1 molar ratio with G-actin, with a dissociation constant of about 10^{-10} M (Engel et al., 1977). It has long been known that the binding of ATP to its binding site on G-actin and the binding of divalent metal ion to the high-affinity metal binding site affect each other (Tonomura & Yoshimura, 1962; Strohman & Samarodin, 1962; Kuehl & Gergely, 1969). Treatment of G-actin with EDTA or Dowex 50 to remove divalent metal ion from the high-affinity site results in a dramatic increase in the dissociation rate constant for ATP leaving G-actin and subsequent irreversible denaturation of the protein (Strzelecka-Golaszewska & Drabikowski, 1967; Strzelecka-Golaszewska et al., 1974; Waechter & Engel, 1977). Alternately, the abstraction of bound ATP by Dowex causes a more rapid dissociation of divalent metal ion from the high-affinity site and again protein denaturation (Bárány et al., 1962; Strzelecka-Golaszewska & Drabikowski, 1967). This parallel dissociation of divalent metal ion and ATP indicates that the ATP binding site and high-affinity metal ion binding site interact strongly but does not prove that these two sites are in fact geographically close to each other on the G-actin molecule. The binding constants for ATP to actin in the presence of divalent cation are about 1000 times stronger than in the absence of cation (West, 1971). In the absence of cation, both ADP and ATP have similar binding constants, while in the presence of bound divalent cation, ATP binds

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[1] Abbreviations: K_D, dissociation constant; ESR, electron spin resonance; k_{-1}, dissociation rate constant; Tris, tris(hydroxymethyl)aminomethane; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; EDTA, ethylenediaminetetraacetic acid.

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