Nuclear Protein Modification and Chromatin Substructure. 2. Internucleosomal Localization of Poly(adenosine diphosphate-ribose) Polymerase†

Chandrakant P. Giri,‡ Michael H. P. West, Michele L. Ramirez, and Mark Smulson*

ABSTRACT: Definitive evidence that poly(ADP-Rib) polymerase activity is localized within internucleosomal "linker" regions of HeLa cell chromatin is presented. This evidence was based on the following criteria: the enzyme activity did not coincide with the position of core particles in a sucrose gradient but was displaced to that part of the gradient which is enriched in monomers with linker regions. This was not due to dimer contamination, since resedimentation did not affect the enzyme activity in relation to the monomer. A new method of assaying enzyme activity directly in polyacrylamide gels following the separation of monomers and dimers showed that only dimers and monomers with linker regions contained activity. When dimers were digested, the enzyme activity moved from the dimer to the monomer with linker.

The firm association of a nonhistone protein, namely, poly(ADP-Rib) polymerase,[1] with chromatin (see reviews by Hayaishi and Ueda, 1977; Hilz and Stone, 1976; Smulson and Shall, 1976) offers the opportunity to determine its precise location in the chromatin substructure and to probe basic questions of chromatin structure and function. The basic repeating unit of chromatin, termed a nucleosome (Oudet et al., 1975) or "nu" body (Olins and Olins, 1974), is composed of about 200 base pairs of DNA in association with an octomeric "core" of histones (two copies each of H2A, H2B, H3, and H4). Although the repeat length of DNA is variable in different cell types, the length of DNA in the core particles is invariably 140 base pairs. The remaining 20–60 base pairs are thought to constitute a "linker" between adjacent cores. Histone H1 has been located in this region (Varshavsky et al., 1976; Whitlock and Simpson, 1976). In contrast to the histones, little is known about the distribution of the nonhistone proteins in the chromatin substructure, although their functional significance has been well appreciated (for a review see Stein et al., 1974).

In part 1 (Giri et al., 1978), we presented considerable evidence relating to the importance of chromatin folding in determining which proteins in nucleosomes were modified. From our previous observations (Mullins et al., 1977), we concluded that poly(ADP-Rib) polymerase was probably associated with the linker region in HeLa chromatin. Dimer, trimer, and oligomer nucleosomal fragments were enzymatically active, but mononucleosomes showed very little activity. Varshavsky and his co-workers (Varshavsky et al., 1976; Bakayev et al., 1977), and Todd and Garrard (1977) by two-dimensional electrophoresis, recently showed that it is possible to characterize and isolate a variety of mononucleosomes, including H1-deficient cores and cores with linker region which contain H1. In this study, we have adapted their technique to separate mononucleosomes into various categories, with a view to precisely locating poly(ADP-Rib) polymerase.

Materials and Methods

Supplies were obtained from the same sources as described elsewhere (see preceding paper in this issue). In addition, ϕX174 DNA and *Hae*III restriction endonuclease were gifts from Dr. Jack Chirikjian of this department. HeLa cells were maintained as described in the preceding paper of this issue.

Micrococcal nuclease-digested chromatin was prepared from isolated HeLa cell nuclei according to the low-salt procedure of Noll et al. (1975). Washed HeLa nuclei from 4.8×10^8 cells were suspended in buffer A [50 mM Tris-HCl (pH 7.4), 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, 15 mM β-mercaptoethanol, and 0.2 mM $PhCH_2SO_2F$] at 8×10^7 nuclei/mL. This suspension (6 mL) was made 1 mM with respect to $CaCl_2$, warmed at 37 °C for 4 min, and then digested with micrococcal nuclease at 200 units/mL for 2 min at 37 °C. The resultant nuclease-digested chromatin was isolated and fractionated on linear 5–20% sucrose gradients basically as previously described (Mullins et al., 1977). Poly(ADP-Rib) polymerase activity in the individual or pooled sucrose gradient fractions was assayed in a final volume of 0.5 mL as described earlier (Mullins et al., 1977).

Gel Electrophoresis of DNA. DNA was isolated from the pooled sucrose gradient fractions (Todd and Garrard, 1977) and subjected to electrophoresis (4 °C) in 6% acrylamide native slab gels according to Peacock and Dingman (1967). *Hae*III restriction endonuclease digested ϕX174 DNA fragments were used as molecular weight markers. The DNA size was determined from a plot of log molecular weight vs. relative mobility of these fragments to the bromophenol blue tracking dye.

Gel Electrophoresis of Chromatin. Nuclease-digested chromatin fragments were resolved by electrophoresis at 4 °C in 5% native acrylamide slab gels as described by Varshavsky et al. (1976).

Poly(ADP-Rib) polymerase activity was assayed directly in the chromatin gels by layering a modified assay mix onto the slab. In a final volume of 1.6 mL, this mix contained: 750

† From the Department of Biochemistry, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C. 20007. *Received January 9, 1978; revised manuscript received April 17, 1978.* Supported by National Institutes of Health Grant CA 13195.

‡ Submitted to the Department of Biochemistry in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Biology, University of Rochester, Rochester, N.Y. 14627.

[1] Abbreviations used are: ADP-Rib, adenosine diphosphate-ribose; NAD, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; Cl_3AcOH, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; DTT; dithiothreitol.

0006-2960/78/0417-3501$01.00/0

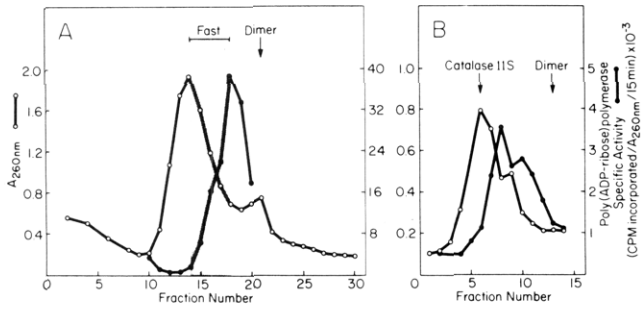

FIGURE 1: Distribution of poly(ADP-Rib) polymerase specific activity between 11S mononucleosomes with and without linker regions. (A) HeLa nuclei were digested with micrococcal nuclease according to the procedure of Noll et al. (1975). The nucleosomes were fractionated by sucrose gradient centrifugation as described under Materials and Methods. The fractions were monitored both for A_{260} (O–O) and for poly(ADP-Rib) polymerase specific activity (●–●) as described. Fractions indicated by the bars were pooled for the experiment described in Figure 1B. Sedimentation is from left to right. (B) Pooled mononucleosomes representing the heavy fraction were concentrated by vacuum dialysis and layered onto a 5-mL gradient containing 8–13% sucrose in 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.2 mM $PhCH_2SO_2F$. Catalase (11S) and purified dimers were run in identical gradients. The gradients were centrifuged for 9 h at 34 000 rpm, at 4 °C, and were fractionated from the top, using a Densiflow. The first 15 fractions were monitored for A_{260} (O–O), and the specific activity of poly(ADP-Rib) polymerase (●–●) was measured. Sedimentation is from left to right. The arrows indicate the positions of the catalase and dimer markers in separate gradients.

mM Tris-HCl (pH 8.0), 7.5 mM DTT, 30 mM $MgCl_2$, 1 μCi of [^{32}P]NAD (0.2 nmol). The gel was then incubated at room temperature (22 °C) for 30 min. Excess mix was removed, and the gel was stained with ethidium bromide (3 μg/mL in 2 mM EDTA, pH 7.6) for 20 min. The gel was photographed with a Polaroid camera using Type 57 film (1 s at F4.5) and was placed in 20% Cl_3AcOH, 5 mM pyrophosphate overnight. The gel was then washed with 5% Cl_3AcOH containing 5 mM pyrophosphate, electrophoresed in a Canalco slab gel destainer for 10 min, and dried onto Whatman 3 MM paper in a Bio-Rad gel dryer. X-ray film was exposed to dried gels for an appropriate time. The autoradiograms and the stained gels were photographed and the negative scanned in a densitometer (ORTEC Inc.).

Results

We have shown that monomers isolated under our digestion conditions are heterogeneous with respect to their DNA length (156–170 base pairs) and with respect to the presence or absence of H1 (Mullins et al., 1977). Whitlock and Simpson (1976) observed that the monomers could be partially fractionated into core and core plus linker particles by sedimentation through a sucrose density gradient. We applied this technique (Figure 1) to the separation of monomers and investigated the distribution of poly(ADP-Rib) polymerase activity across the monomer peak (Figure 1A). There is very little contamination by dimers. The enzyme activity was associated with particles which sedimented more quickly than the bulk of the monomers but more slowly than dimer, whose position is indicated in the figure. There remained the possibility that the activity was a result of "tailing" from the dimer peak, although the specific activity did decline as the dimer peak was approached. This interpretation, however, remained valid as long as the method of separation allowed some mixing to occur. We therefore used three approaches to eliminate the problem. These were resedimentation, DNA size analysis, and gel electrophoresis.

Resedimentation of Monomers. The pooled fractions containing monomers from the heavy side of the peak in Figure 1A were concentrated, dialyzed, and resedimented (Figure 1B). Although some loss in activity was experienced, possibly as a result of the concentrating procedure, the specific activity continued to be associated with those particles which sedimented more slowly than the dimer. Very little of the material which absorbed at 260 nm sedimented to the dimer position, and the enzyme activity there was minimal. Fractions corresponding to the light- and heavy-monomer regions in a *comparable* gradient were analyzed with respect to the length of DNA in each fraction (Table I) and to their mobility in nondenaturing "chromatin" gels (Figure 2).

DNA Size Analysis. DNA from the light, heavy, and total peak fractions was isolated and separated by electrophoresis in a 6% acrylamide gel according to the procedure of Peacock and Dingman (1967). The total peak contained DNA ranging

TABLE I: Approximate Molecular Weights and Sizes of DNA[a] Fragments Isolated from Pooled Sucrose Gradient Fractions Corresponding to Nucleosome Monomer Region.

monomer peak fractions pooled	no. of bands	extrap mol wt range ($\times 10^4$)	no. of base pairs range (mol wt/668)
total	1 (br)	9.0–11.5	136–174
light	3	8.7–9.0	132–136
		9.4–9.6	142–145
		9.8–10.5	148–159
heavy	2	8.6–8.8	130–133
		9.2–11.0	139–167

[a] DNA isolated from the pooled sucrose gradient fractions by the method of Todd and Garrard (1977) was subjected to electrophoresis in native 6% acrylamide gels according to Peacock and Dingman (1967). The approximate size of the DNA fragments was determined from a plot of relative mobility vs. molecular weight of ϕX174 DNA fragments produced by digestion with *Hae*III restriction endonuclease as described under Materials and Methods.

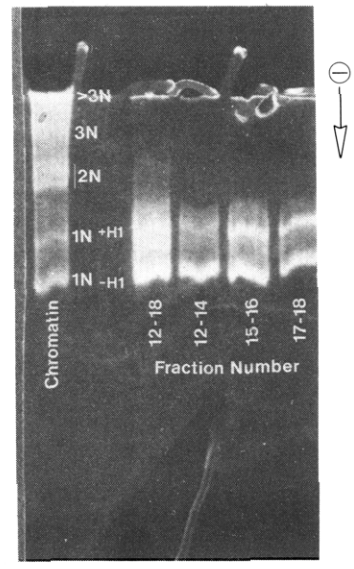

FIGURE 2: Polyacrylamide gel electrophoresis of micrococcal nuclease digested chromatin fragments. Chromatin fragments were fractionated by sucrose gradient centrifugation as described under Materials and Methods under similar conditions to those shown in Figure 1A. The pooled fractions, corresponding to nucleosome monomers, were directly subjected to electrophoresis in a "native" 5% polyacrylamide slab gel according to the method of Varshavsky et al. (1976). The pooled fractions corresponding to total monomers (numbers 12–18) were concentrated by vacuum dialysis prior to electrophoresis. The unfractionated chromatin fragments were electrophoresed as markers to display the relative positions in the gel corresponding to monomer (1 N) with and without H1, dimer (2 N), trimer (3 N), and oligomer (>3 N). The electrophoresis was performed at 200 V for approximately 2 h at 4 °C.

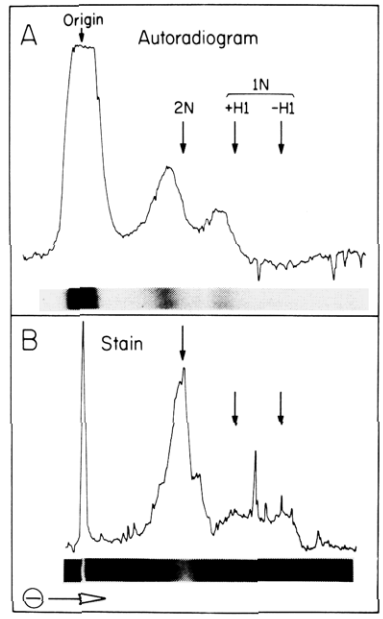

FIGURE 3: Poly(ADP-Rib) polymerase activity assayed directly (in situ) following electrophoresis of an isolated preparation of nucleosome dimers and monomers in a native polyacrylamide slab gel. Approximately 40 μL of a dimer preparation (Figure 3A) containing monomer as a minor component was assayed for poly(ADP-Rib) polymerase activity (in situ) following its electrophoresis in a native polyacrylamide gel as described under Materials and Methods. An autoradiogram was prepared and scanned as described under Materials and Methods.

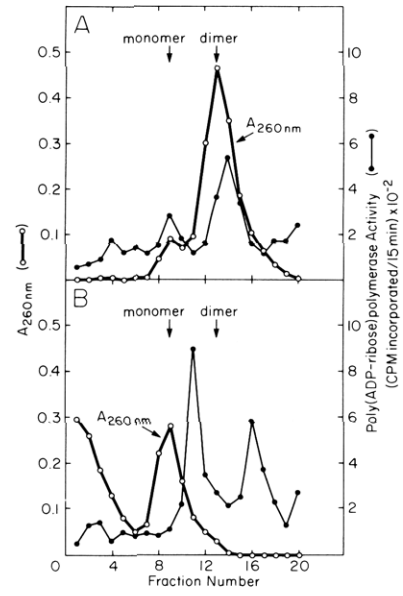

FIGURE 4: Redigestion of an isolated nucleosome dimer. Nuclease-digested chromatin isolated by the "low salt" method of Noll et al. (1975) was fractionated by sucrose gradient centrifugation as described under Materials and Methods. The appropriate fractions corresponding to the nucleosome dimer peak were pooled and concentrated by vacuum dialysis against 10 mM Tris-HCl (pH 8.0), 0.2 mM NaEGTA, and 0.15 M NaCl at 4 °C. The 0.5-mL concentrated "dimer" preparation (A_{260} = 1.28) was adjusted with respect to 0.7 mM $CaCl_2$, warmed at 37 °C for 30 s, and then redigested with micrococcal nuclease (100 units/mL) at 37 °C for 2 min. The reaction was stopped by chilling in an ice bath and by the addition of NaEGTA, pH 8.0 (5 mM). The control dimer preparation (A_{260} = 1.28) was processed identically as the redigested sample, except NaEGTA was added before the nuclease and kept at 4 °C at all times. Both control (A) and the redigested (B) preparations were layered onto each of two 12-mL sucrose gradients in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM NaCl, and 0.2 mM $PhCH_2SO_2F$, and centrifuged at 38 000 rpm for 12 h in a Beckman SW 40 rotor. The gradients were then fractionated and monitored for A_{260} (○) and poly(ADP-Rib) polymerase activity (●) as previously described (Mullins et al., 1977).

in length from 136 to 174 base pairs, indicating a heterogeneous population of monomers. Both light and heavy fractions contained core particles (130–145 base pairs), and core plus linkers (148–167 base pairs); however, the late fractions were enriched in the latter type of particle. In agreement with these results, electrophoretic analysis of the complete peak (Figure 2) showed a heterogeneity in the monomer fraction, with two prominent bands. Fractions from the heavy side of the peak (Fractions 15–18) show an enrichment for the more slowly migrating monomer, which contains an attached linker region.

In part 1 it was shown that the core particles generated in our experiments have the histones H2A, H2B, H3, and H4, and the cores plus linkers have, in addition, H1; we have also characterized the proteins in these particles which act as acceptors for poly(ADP-Rib).

Assay for Polymerase Activity Directly in Gels following Separation of Chromatin Fragments. The experimental evidence strongly supports the conclusion that only cores with linker regions attached had activity for poly(ADP-Rib) polymerase. To provide definitive evidence, it was decided to separate monomers of different types completely by electrophoresis in a "chromatin" gel system (Varshavsky et al., 1976) and then to assay directly in the gel for enzyme activity. This procedure has been carried out successfully for DNA and RNA polymerase in polyacrylamide gels (Beckman and Frankel, 1976), although it was necessary to imbed DNA in the gel. This was unnecessary for poly(ADP-Rib) polymerase, since the chromatin fragments contain both DNA and acceptor proteins which are required for the functioning of the enzyme. Control experiments indicated that this method could also be applied to detect activity in dimers and trimers, and that thymidine, a potent inhibitor of poly(ADP-Rib) polymerase, markedly reduced the enzymatic incorporation of labeled NAD into the gel. This technique was utilized to identify which of the two types of monomer exhibited enzyme activity (Figure 3). In this experiment, a preparation containing monomer and dimer was separated on the gel and assayed as described under Materials and Methods. By this means, dimer, which we know to have activity, could be used as a marker. Aggregated material, which contained high activity, was seen at the origin; this is a limitation of the method and it is difficult to eliminate. However, enzyme activity (Figure 3A) was detected in the dimer and in the monomer which contained the linker region. No activity was seen in core particles. The activity appeared in particles which migrated more slowly than the bulk of the particles in the dimer and monomer fractions, suggesting a subpopulation. Varshavsky et al. (1976) reported three bands of dimers, corresponding to particles containing zero, one or two molecules of H1. Heterogeneity with respect to poly(ADP-Rib) polymerase has been confirmed in this study.

Redigestion of Dimer. If poly(ADP-Rib) polymerase is located in the linker regions, redigestion of a dimer preparation should result in the movement of activity to the monomers containing those regions. The results of such an experiment are shown in Figure 4. It is shown that conversion of dimers into monomers plus linkers and core particles does occur and that the enzyme activity migrates with the former type of particles. In addition, some material migrated more quickly than the undigested dimer, showing enzyme activity. This contained both DNA and acceptor proteins, since it was not necessary to add these in order to detect the activity. It probably consisted of aggregated chromatin, although its exact significance remains as yet unknown.

Discussion

Previous results (Mullins et al., 1977) had indicated that poly(ADP-Rib) polymerase was located in the linker regions of chromatin, between nucleosomes. The present studies, which are much more detailed, confirm and extend our previous conclusions. A new method for assaying poly(ADP-Rib) polymerase in polyacrylamide gels has been developed. The evidence supporting the internucleosomal location of the enzyme is based on the following criteria: (a) enzyme activity did not coincide with core particles but was displaced to the sucrose gradient fractions which were enriched in monomers with linker regions; (b) this activity was not due to dimer contamination, since resedimentation did not affect the enzyme activity in relation to the monomer peak; (c) enzyme activity in polyacrylamide gels was only associated with particles which had linker regions, i.e., dimers and the more slowly migrating monomers; and (d) when dimers were further digested the enzyme activity moved from the dimer to the monomer with linker.

It was also concluded that a subpopulation of monomers with linker which contained the enzyme was present and that this migrated more slowly in the polyacrylamide gel. Heterogeneity in the monomers with respect to the presence of H1 has already been reported by Varshavsky et al. (1976) and Todd and Garrard (1977) have shown a precursor relationship between different classes of monomers. This study shows a heterogeneity amongst monomers containing linker region with respect to enzyme activity, and preliminary results from our laboratory indicate that nucleosomes containing the enzyme can be selected and purified using an affinity column technique (West and Smulson, manuscript in preparation).

Since H1 is a major acceptor amongst histones for chromatin-bound poly(ADP-Rib) polymerase (see reviews by Hayaishi and Ueda, 1977; Hilz and Stone, 1976), and since a dimer of H1 cross-linked by poly(ADP-rib) can be isolated (Stone et al., 1977), experiments are in progress to determine whether the association of H1 with the linker region is a prerequisite for the endogenous enzyme. Preliminary results (Giri and Smulson, unpublished observations) indicate, however, that HeLa chromatin, extracted with 0.6 M NaCl to dissociate H1, still retained activity. In fact, the specific activity of the enzyme was simulated three- to four-fold from the control level.

In part I (Giri et al., 1978), considerable data regarding the ADP ribosylation of proteins have been presented, and these can be summarized as follows: (1) Histone H1 is one of the major acceptors for chromatin-bound poly(ADP-Rib) polymerase. (2) The core histone, H4, is either not modified or its linkage to ADP ribose is extremely labile under the conditions used here. (3) The remaining core histones, and in particular H2B, are modified to an appreciable extent.

The data reported in part I clearly emphasize the importance of chromatin conformation in determining which proteins become modified. Recent observations in our laboratory indicate that the specific activity of the enzyme increases with increasing nucleosome number up to a limit of six to nine nucleosomes (Butt et al., 1978). This may suggest either that the activity is influenced by the folding of chromatin or that modification helps to stabilize these structures. In any case, it is clear that the location of the enzyme between nucleosomes does not prevent its modification of core histones.

The studies reported here are being extended in order to provide answers relating to the function of the enzyme. Our previous studies (Mullins et al., 1977) revealed that the ADP-ribosylating system is located preferentially within transcriptionally active or more extended forms of chromatin. It has also been reported that the introduction of single-strand nicks in the DNA, either in vitro by DNase I (Miller, 1975; Smulson et al., 1977) or in vivo by certain alkylating drugs, such as methylnitrosourea (Smulson et al., 1977) or bleomycin (Miller, 1977), results in an activation of poly(ADP-Rib) polymerase. These reports suggest a possible involvement of the enzyme in transcription and DNA repair. The characteristics of the enzyme itself are also under investigation. Hayaishi and Ueda (1977) suggested the presence of at least two enzymes in the system: a ligase which adds the initial ADP-ribose moiety to the acceptor and an elongating enzyme which synthesizes the chain. Preliminary experiments support this hypothesis (West and Smulson, manuscript in preparation).

In conclusion, the work described in these papers, in addition to revealing many aspects of the ADP-ribosylating system, also provides techniques which can be utilized to investigate, in a precise way, the function or functions which this enzyme, and possibly other nuclear enzymes, carry out in the cell.

Acknowledgments

The authors are indebted to Drs. Leonard H. Cohen, Martin A. Gorovsky, and Robert T. Simpson for helpful discussions and for critically reviewing the manuscript.

We thank Drs. Robert Suhadolnik for providing us with [α-^{32}P]NAD and Jack G. Chirikjian of this department for ϕX174 DNA and *Hae*III restriction endonuclease used in the present experiments.

References

Bakayev, V. V., Bakayeva, T. G., and Varshavsky, A. J. (1977), *Cell 11,* 619.

Butt, T. R., Brothers, J. F., Giri, C. P., and Smulson, M. E. (1978) *Nucleic Acids Res. 5* (in press).

Giri, C. P., West, M. H. P., and Smulson, M. (1978), *Biochemistry* (preceding paper in this issue).

Hayaishi, O., and Ueda, K. (1977), *Annu. Rev. Biochem. 46,* 95.

Hilz, H., and Stone, P. (1976), *Rev. Physiol. Biochem. Pharmacol. 76,* 1.

Miller, E. G. (1975), *Biochim. Biophys. Acta 395,* 191.

Miller, E. G. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 3351.

Mullins, D. W., Jr., Giri, C. P. and Smulson, M. (1977), *Biochemistry 16,* 506.

Noll, M., Thomas, J. O., and Kornberg, R. D. (1975), *Science 187,* 1203.

Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330.

Oudet, P., Gross-Bellard, M., and Chambon, P. (1975), *Cell 4,* 281.

Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6,* 1818.

Smulson, M., and Shall, S. (1976), *Nature (London) 263,* 14.

Smulson, M., Schein, P., Mullins, D. W., Jr., and Sudhakar, S. (1977), *Cancer Res. 37,* 3006.

Stein, G. S., Spelsberg, T. C., and Kleinsmith, L. J. (1974), *Science 183,* 817.

Stone, P. R., Lorimer, W. S., III, and Kidwell, W. R. (1977), *Eur. J. Biochem. 81,* 9.

Todd, R. D., and Garrard, W. T. (1977), *J. Biol. Chem. 252,* 4729.

Varshavsky, A. J., Bakayev, V. V., and Georgiev, G. P. (1976), *Nucleic Acids Res. 3,* 477.

Whitlock, J. P., Jr., and Simpson, R. T. (1976), *Biochemistry 15,* 3307.