ULTRAVIOLET INACTIVATION AND MISCODING OF IRRADIATED R17-RNA IN VITRO

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SUMMARY

Ultraviolet irradiation of the single-stranded Coliphage R17 leads to inactivation of the in vitro messenger activity of its RNA. Uridine-photohydration is the major reaction occurring in the RNA under these conditions. At a residual activity of the synthetase gene of 37 per cent, there are 11.2 uridine-photohydrates present in the total R17 genome, while only 5.3 photohydrates are necessary to decrease the translation of the coat gene to the same value. De novo incorporation of histidine into the coat protein is observed with R17-RNA from ultraviolet-irradiated phage, indicating that uridine-photohydrates can miscode as cytidine in an in vitro translation system.

Despite a considerable amount of work, the question whether uridine-photohydrates (6-hydroxy-5,6-dihydrouridine) in a messenger miscode for cytidine has been left in a controversial state. Grossman (1) has tested ultraviolet-irradiated polyuridylic acid in an in vitro protein-synthesizing system and observed the dose-dependent de novo incorporation of serine (codon UCX) and a decrease in the incorporation of phenylalanine (codon UUU). Since the serine incorporation could be eliminated by preheating the polymer, it was concluded that uridine-photohydrates were responsible for the miscoding. Singer and Fraenkel-Conrat (2), however, were unable to confirm this finding. Since the irradiated polyuridylic acid in these experiments contained both photodimers and photohydrates at levels which were not accurately determined, these results have to be valued with caution. No miscoding of 5,6-dihydrouridine, a close analogue of the photohydration product of uridine (6-hydroxy-5, 6-dihydrouridine), was observed by Rottman and Cerutti (3) in studies of the template activity of copolymers of 5,6-dihydrouridine and uridine, both in an in vitro protein-synthesizing system and in the binding of

aminoacyl-tRNA to ribosomes. Ottensmeyer and Whitmore (4) found an increase in histidine incorporation into peptide with irradiated poly U and a corresponding decrease in tyrosine incorporation in separate experiments. The same authors also reported the binding of arg-tRNA and thr-tRNA to ribosomes in the presence of the irradiated trinucleotide UpUpU and concluded that uridine under these conditions miscodes as cytidine. Finally, they observed the phenotypic reversion of an amber mutant of phage λ when irradiated with a germicidal lamp five minutes after infection of a nonpermissive E. coli host. From the increased burst size after irradiation, the authors concluded that the uridine-photohydrates are coding as cytidine to permit the synthesis of a complete, active protein (5).

We have used the following approach to clarify this issue. Advantage was taken of the fact that the coat protein of Coliphage R17 lacks the amino acid histidine (codon CA^{U}_{C}), while it contains four tyrosine residues (codon UA^{U}_{C}) (6). We postulated that de novo incorporation of histidine into the coat protein should be observed in a translation system using ultraviolet-irradiated R17-RNA if uridine-photohydrates in the first position of the tyrosine codon are indeed misread as cytidine. This system has the advantage that a natural messenger is used rather than a synthetic polymer or oligonucleotide, that conditions were available for the production of RNA-containing photohydrates but no detectable photodimers, and that the extent of uridine-photohydration could be chemically determined by the reductive assay described earlier (7). It was found that uridine-photohydrates in R17-RNA both deactivate the messenger and miscode as cytidine with high efficiency.

Inactivation of Coat Protein and Synthetase Genes

Irradiation of intact R17 bacteriophage at 280 nm produced uridine-photohydrates and no detectable dimers, as reported by Remsen, et al. (8). Cytidine-photohydrates are formed with 10-15 times lower efficiency in R17-RNA (9). It follows that the biological effects observed with RNA prepared from irradiated phage are most likely attributable to uridine photohydration,

but a contribution by some unknown additional photoproducts cannot be fully excluded.

Intact R17 bacteriophage was irradiated at 280 nm, as described in the legend of Fig. 1, and the RNA extracted following irradiation was used as a messenger in an in vitro translation system. The incorporation of valine into protein was used as a measure of total messenger activity and of specific gene deactivation. The proteins were separated by electrophoresis on SDS-polyacrylamide gels. Since the A-protein-deficient mutant, R17 amA_9, was used, only two major proteins, namely coat protein and synthetase, were synthesized. As shown in Fig. 1, single-hit kinetics were obtained for the inactivation of total messenger activity and of the synthetase and coat-protein genes. The same inactivation curves were obtained whether valine was labeled with tritium or carbon-14. The coat-protein gene was found to be inactivated at a faster rate than the RNA-synthetase gene, while the overall rate approached an average between the two, despite the fact that the target size of the synthetase gene must be more than threefold larger from comparison of the molecular weights: the coat protein is 13,750 daltons, while the synthetase is 60,000 daltons (6,12). There is no obvious explanation for the different ultraviolet sensitivity of the two genes.

Eker and Berends (13) have suggested that ultraviolet inactivation of the messenger activity of polyuridylic acid is due to photodimers rather than to photohydrates. This conclusion is questionable in view of the results reported here with R17-RNA. A comparison with our earlier results on the ultraviolet-induced loss of infectivity of intact R17 phage and R17-RNA (8, 14) indicates that over five times more uridine-photohydrates per RNA molecule are needed to decrease the messenger activity to 37% than to decrease the infectivity to the same amount. It follows that deactivation of the messenger by uridine photohydration is not primarily responsible for the lethal effect of ultraviolet light on R17. It is more likely that photohydrates interfere with the formation of the double-stranded replicative form of the phage.

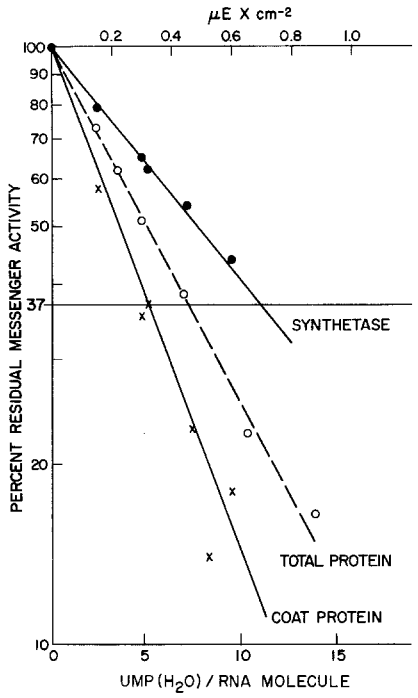

Fig. 1. Inactivation of messenger activity of R17-RNA by ultraviolet irradiation of the phage at 280 nm. The Coliphage, R17 amA_9 initially obtained from A. Weiner, Harvard University, was irradiated at 280 nm in 10^{-3} M EDTA, pH 8, at room temperature in a concentration of 0.5 mg phage per ml, with a Schoeffel monochromator with a half-band width of approximately 1 nm. Following irradiation, the RNA was extracted with SDS-phenol at 0° and precipitated in ethanol followed by a cold ether wash of the pellet. RNA recovery was quantitated for each sample using a value for the specific absorption of 23 per mg of RNA per ml at 260 nm.

In vitro protein synthesis was carried out with, in μmoles per ml: Tris-HCl, pH 7.5, 50; Mg-acetate, 12; KCl, 30; NH_4Cl, 30; 2-mercaptoethanol, 5; ATP, 1; GTP, 0.2; E. coli tRNA (commercial), 200 μg; phosphoenol pyruvate, potassium salt, 5; pyruvate kinase (lyophilized, Worthington), 20 μg; amino acids, unlabeled, 0.05; radioactive valine and histidine; plus S30 extract of E. coli K12 Hfr RNase⁻, and R17-RNA, 300-400 μg (10,11). The amount of S30 extract added was optimalized for each preparation with unirradiated, freshly extracted R17-RNA amA9. Final specific activities in the reaction mixture were: ^{3}H-valine, 417, or ^{14}C-valine, 86-128 (New England Nuclear). After incubation at 36°C for 30 minutes with gentle agitation, the samples were immediately chilled and 20 μl aliquots were removed for determination of total protein synthesis. The remainder of each sample was digested with pancreatic RNase (Worthington), denatured with SDS, and dialyzed against 0.01 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS and 0.1% 2-mercaptoethanol (11). 200 μl aliquots were submitted to overnight electrophoresis in 12 cm 10% polyacrylamide gels in 0.1 M sodium phosphate buffer, pH 7.2, --0.1% SDS, at 3 mamp per gel. The gels were sliced, dissolved, and counted.

The total radioactivity in valine under each peak on polyacrylamide gels was used to calculate the decay of messenger activity for the synthetase and coat-protein genes. Total protein synthesis was determined by collection of the hot TCA precipitate of the incorporation mixture on membrane filters (B-6 Bac-T-Flex, Schleicher and Schuell) and counting in toluene scintillation solution (10).

Miscoding of Uridine-Photohydrates in R17-RNA

Double-labeling experiments were carried out to study the incorporation of histidine into coat protein in the *in vitro* translation of R17-RNA extracted from ultraviolet-irradiated phage. As discussed in the introductory paragraph, *de novo* incorporation of histidine into the coat protein is expected if uridine-photohydrates miscode for cytidine. The *in vitro* translation system was as described in the legend to Fig. 1 except that [^{14}C]-valine and [^{3}H]-histidine were both added to each reaction mixture. Valine was used as reference amino acid, since it is abundant in the coat protein and allowed the computation of the amount of coat protein produced at a given ultraviolet dose. In the initial experiments, the labeled proteins were applied to gels directly. Subsequently, the proteins synthesized *in vitro* were first chromatographed on a Sephadex G-100 column (in 0.05 M phosphate buffer, pH 7.2, containing 8 M urea) to remove the major portion of the synthetase and small protein fragments before the acrylamide-gel electrophoresis. The Sephadex column was calibrated with unlabeled coat protein isolated from purified R17 amA_9 phage. Gel patterns now showed a single sharp protein peak with the mobility of R17 coat protein. Contamination of the peak with fragments originating from the R17 synthetase protein with properties identical to those of the coat protein appears unlikely but cannot be rigorously excluded. The three fractions from the gels containing the radioactivity peak at the location of the coat protein were used to calculate the data given in Fig. 2. The ratio of histidine over valine in the coat protein is plotted as a function of the ultraviolet dose and the level of uridine-photohydration. A linear increase of this ratio is observed with dose. The same result was obtained when the radioactive labels of valine and histidine were exchanged (*i.e.*, ^{3}H -valine and ^{14}C -histidine were used). Samples of coat protein which was synthesized in response to irradiated and unirradiated R17-RNA were hydrolyzed in 6 N HCl and the digests analyzed by thin-layer chromatography. All the radioactivity cochromatographed with authentic valine and histidine

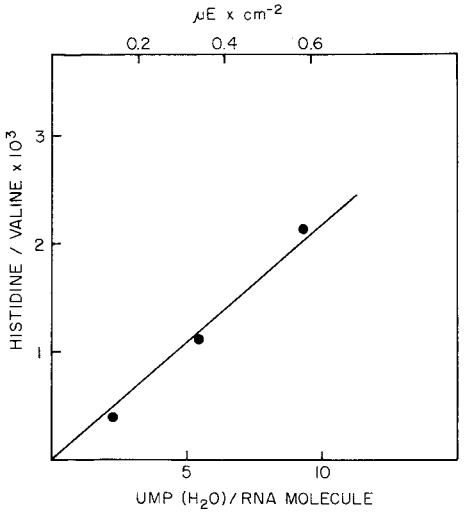

Fig. 2. De novo incorporation of histidine into the coat protein as a function of uridine-photohydration in R17-RNA. Incorporation of [^{14}C]-valine and [^{3}H]-histidine into R17 coat protein in response to RNA extracted from ultraviolet-irradiated phage was studied using the experimental conditions described in the legend to Fig. 1. The reaction mixture obtained in the in vitro incorporation experiment was first chromatographed on a Sephadex G-100 column. The amount of [^{14}C]-valine and [^{3}H]-histidine incorporated was calculated from the radioactivity in the coat-protein region of the polyacrylamide gels after correction for overlap between the two radioactive labels and for counting efficiency. The ratio of histidine over valine incorporated is plotted as a function of the uridine-photohydrate content of the RNA and the ultraviolet exposure. The data were corrected for the value of hist/val found with unirradiated R17-RNA due to background incorporation of histidine. Analogous results were obtained when the isotopes of the two amino acids were reversed. Specific activities of the amino acids: [^{14}C]-valine, 128, and [^{3}H]-histidine, 3852.

markers. It is concluded that uridine photohydrates can miscode as cytidine in the in vitro translation of the coat protein gene of R17. It is not possible to calculate a miscoding efficiency for uridine-photohydrates from our data, since the photoproducts are probably not introduced in a nonrandom fashion (7) and since the photohydrates cause deactivation as well as miscoding.

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