Because the global mean temperature increase is linearly proportional to the CO_2 forcing, a figure similar to Fig. 1 but showing the ratio of the temperature increase to the CO_2 forcing would be far more informative because this normalized temperature increase would remove forcing differences and so isolate differences caused by feedback processes (*12*).

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Photocrosslinking of 5-Iodouracil–Substituted RNA and DNA to Proteins

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5-Iodouracil–substituted RNA and DNA were crosslinked regiospecifically to associated proteins in yields of 70 to 94% of bound nucleic acid. Irradiation of the iodouracil chromophore with monochromatic, long-wavelength ultraviolet radiation (325 nanometers) eliminates excitation of other nucleic acid and protein chromophores. The combination of high crosslinking yields, excellent specificity, and elimination of photodamage to other chromophores represents an important advance toward the precise identification of contacts in nucleoprotein complexes.

During the past two decades 5-bromodeoxyuridine (BrdU) has been incorporated into DNA to enhance photosensitivity (*1*). Photoreactions include formation of single- and double-strand breaks, alkali labile bonds, and crosslinks to associated proteins. Of particular interest has been photocrosslinking to associated proteins to establish point contacts as a method for partially defining the structure of nucleoprotein complexes. Low-level 5-bromodeoxyuridine–DNA (BrdU-DNA) photocrosslinking to associated proteins has been reported (*2–4*), and in two cases point contacts have been established (*3*, *4*). 5-Bromouridine–RNA (BrU-RNA) photocrosslinking to associated proteins has also been reported (*5*, *6*), including R17 bacteriophage coat protein to singly BrU-substituted hairpin RNA 1 (Fig. 1) (*6*).

Creation of a photoreactive nucleic acid chromophore by replacement of the methyl group of thymine with a bromine is attractive because the van der Waals radius of bromine (1.95 Å) is similar to the size of a methyl group (2.0 Å). The van der Waals radius of iodine is 2.15 Å, only 8% larger than the methyl group. A single substitution of IdU for T also does not appreciably disturb the protein-DNA complex studied here (Fig. 1). Even the single BrU or IU for U substitution in the binding site for bacteriophage R17 coat protein (RNA 1 or RNA 2, respectively) does not interfere with binding. In fact, the bacteriophage R17 coat protein binds RNA 2 as well as RNA 1 and better than its uridine equivalent (RNA 3).

RNAs 1 and 2 both undergo photocrosslinking to the R17 coat protein; yields as a function of time of irradiation at 308 nm with a XeCl excimer laser are compared in Fig. 2. Crosslinking of the IU–RNA 2–R17 coat protein complex leveled off at 80% in less than 5 min of irradiation, whereas the corresponding BrU–RNA 1–R17 coat protein crosslinking leveled off at 40% after 15 min of irradiation. The two crosslinked nucleoprotein complexes showed identical electrophoretic migration in both SDS- and urea-denaturing polyacrylamide gels (*7*). Crosslinking to Tyr^{85} of the coat protein was established with BrU–RNA 1 (*8*). The lower crosslinking yield with BrU-RNA was a consequence of protein damage (*6*). Crosslinking as a function of photons absorbed indicated that the quantum yield for crosslinking of BrU–RNA 1 is actually about twice that of IU–RNA 2 (0.014 as compared with 0.006, respectively) (*9*). In spite of the lower quantum yield with IU substitution, a higher crosslinking yield was obtained as a result of the sevenfold higher absorption probability of the IU chromophore at 308 nm. Hence, a high level of photocrosslinking was achieved before protein damage. Placement of IU at other positions in the hairpin, as described previously for BrU substitution (*6*), did not yield appreciable photocrosslinks or RNA damage.

Having established the superiority of the iodouracil chromophore upon excitation at 308 nm, we tried excitation at a longer wavelength where other nucleoprotein chromophores do not absorb. The photocrosslinking yield of IU–RNA 2 to the R17 coat protein as a function of irradiation time with monochromatic, 325-nm light from a helium cadmium (HeCd) laser is shown in Fig. 3 (*10*).

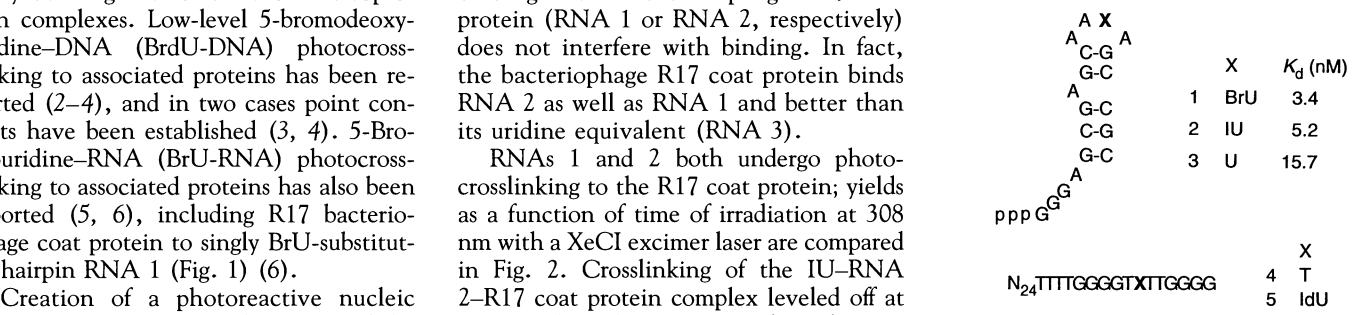

Fig. 1. Structures of the R17 bacteriophage RNA hairpins 1, 2, and 3 (*14*) and the *Oxytricha nova* telomeric DNA oligonucleotides 4 and 5 (*15*) (N_{24} represents 24 nucleotides of nontelomeric DNA). The dissociation constants of the RNA-protein complexes were determined with a nitrocellulose filter retention assay (*16*). For each experiment the data points were fit to a noncooperative binding curve and the dissociation constant (K_d) calculated. The telomeres of the ciliated protozoan *O. nova* have 3′ single-stranded $(T_4G_4)_2$ extensions bound by a heterodimeric protein.

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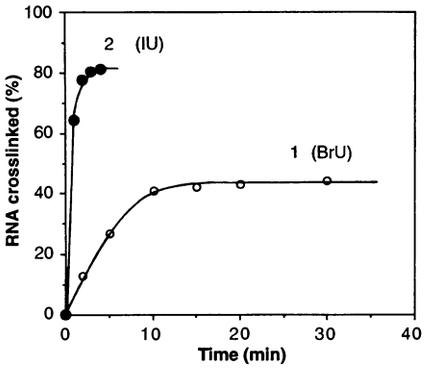

Fig. 2. Photocrosslinking yields of RNA hairpins 1 and 2 to the R17 bacteriophage coat protein as a function of time of irradiation with a XeCl excimer laser at 308 nm (*17*). Crosslinking of 5-bromouridine–containing RNA 1 maximizes at about 40% because of competitive photodamage to the coat protein, which inhibits binding to the RNA (*8*).

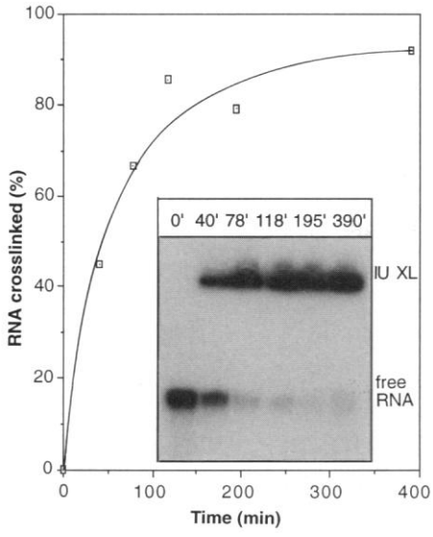

Fig. 3. Photocrosslinking of IU–RNA 2 to the R17 coat protein with monochromatic emission at 325 nm as a function of time. XL marks the photocrosslinked RNA. The power output of the Omnichrome HeCd laser (model 3074-40M325) was 37 mW, and the total beam of diameter 3 mm was incident on the stirred sample in a 1-cm square cuvette. To increase excitation per unit time, the beam was reflected back through a different region of the sample with a dielectric-coated concave mirror. Crosslinked and uncrosslinked RNA were separated by PAGE as shown (**inset**), and the yields were quantitated with the Phosphor Imager (Molecular Design). With monochromatic irradiation at 325 nm, high-yield crosslinking occurs without photodamage to the R17 coat protein (*10*) (also see text). Irradiation of BrU–RNA 1–R17 coat protein complex at 325 nm does not lead to crosslinking, because the 5-bromouracil chromophore is transparent at 325 nm.

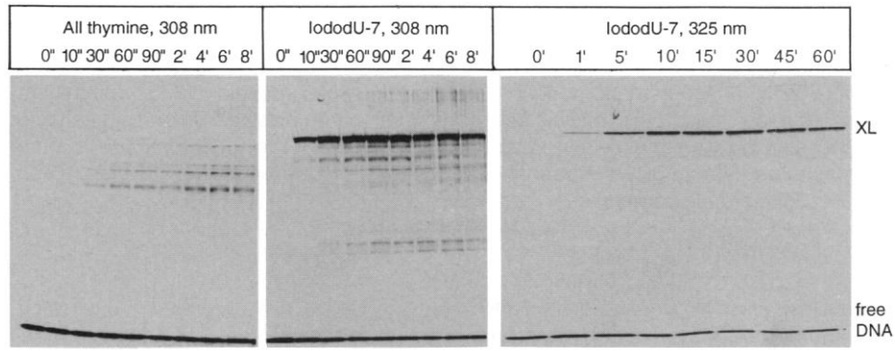

Fig. 4. An SDS-polyacrylamide gel displaying irradiation products of reconstituted *Oxytricha* telomeres as a function of irradiation time. Polypeptide subunits α and β were incubated at 3 and 6 μM, respectively, with 1 μM ^{33}P-labeled DNA 4 or DNA 5 in 10 mM tris (pH 7.5), 20 mM NaCl and irradiated at 308 or at 325 nm. The designation XL is for the major photocrosslink (see text).

Crosslinking yields as high as 94% were achieved (*11*). Furthermore, in a separate control experiment the R17 coat protein was similarly irradiated in the absence of the RNA at 325 nm with an even higher dose, and this resulted in no change in its binding to the RNA as measured by a nitrocellulose filter binding assay.

We also investigated the effect of a single IdU substitution within a single-stranded DNA-protein complex, the telomere of the ciliate *Oxytricha*. A substitution of IdU for T in DNA 4 led to enhanced, regiospecific crosslinking at a yield of 70% (Fig. 4). The unsubstituted (DNA 4) nucleoprotein complex produced several products on irradiation at 308 nm; each product represents a single, specific crosslinking event between protein and DNA (*12*). Many of the low-abundance photocrosslinked forms resulting from irradiation at 308 nm are equivalent for DNAs 4 and 5 and hence result from excitation of non-IdU chromophores.

Irradiation of the IdU-substituted (DNA 5) nucleoprotein complex at 325 nm produced a marked effect: At this longer wavelength, where IdU is the exclusive absorbing chromophore in the nucleoprotein complex, a single major photoproduct is seen (Fig. 4). Crosslinking to His^{292} of the telomere protein α subunit was established with the equivalent BrdU-substituted DNA (*13*).

The yields of photocrosslinking of RNA 2 and DNA 5 to their respective binding proteins are exceptionally high, but still less than 100%. Incomplete photocrosslinking may result from the nucleic acids existing in primary and secondary conformations with at least one secondary conformation unreactive and with the time constant for equilibration of the conformations large relative to the irradiation time. Another possible reason for substoichiometric photocrosslinking is the less than 100% IU or IdU incorporation during nucleic acid synthesis. In fact, the 5-iodouridine triphosphate used in the synthesis of RNA 2 was only 95% pure. Further, in a model experiment 20% of the iodouracil was transformed to a chromophore transparent above 300 nm on treatment with ammonium hydroxide, as is used in the deprotection step of the DNA synthesis protocol.

The value of a high protein–nucleic acid crosslinking yield lies not only in the ease of obtaining pure material for sequencing but also in the certainty that the crosslink is formed within the primary conformation of the complex. A low crosslinking yield would be misleading if it represented photoreaction of a minority complex that happened to photocrosslink efficiently. Also of concern are competitive photoprocesses of the protein or the nucleic acid (or both) that might affect subsequent crosslinking or sequencing (or both).

We conclude that iodouracil substitution, coupled with HeCd laser excitation, is a superior technique for establishing contact points in nucleoprotein complexes. Because of the long wavelength light used, it also has potential for photocrosslinking nucleoprotein complexes in vivo. We further conclude that iodouracil substitution coupled with XeCl laser excitation leads to substantial crosslinking yields after only 10 s of irradiation. Although photocrosslinking at 308 nm also gives some secondary products, the short irradiation time might be useful for studying the kinetics of complex formation or molecular motion within the nucleoprotein complex.

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7. The mechanism for photocoupling of the 5-iodouracil chromophore to amino acid residues has not been established, but it may be similar to that of the 5-bromouracil chromophore [T. M. Dietz, R. J. von Trebra, B. J. Swanson, T. H. Koch, *J. Am. Chem. Soc.* **109**, 1793 (1987)]. A model study was performed to establish similarity between 5-iodouracil and 5-bromouracil photocrosslinking. Irradiation of an aqueous solution of iodouracil and *N*-acetyltyrosine *N*-ethylamide at 308 nm gave a photoadduct identical to that from irradiation of bromouracil and *N*-acetyltyrosine *N*-ethylamide [T. M. Dietz and T. H. Koch, *Photochem. Photobiol.* **46**, 971 (1987)].
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9. A previous measurement of the quantum yield of photocrosslinking of RNA 1 to the R17 coat protein was lower (*6*). Our technique for performing this measurement has improved substantially, and we believe the new value to be correct.
10. Emission from a HeCd laser is advantageous for crosslinking because at 325 nm absorption by other chromophores in the nucleoprotein complex such as thymine, guanine, indole, and phenol is not observed. In the region of 325 nm only the 5-iodouracil chromophore absorbs appreciably; the molar extinction coefficient is 1.63×10^5 cm^2/mol. For comparison, bromouracil and iodouracil absorb at 308 nm with extinction coefficients of 3.85×10^5 and 2.64×10^6 cm^2/mol, respectively. The HeCd laser also eliminates the possibility of two-photon excitation because it is a continuous wave laser as opposed to the pulsed XeCl excimer laser.
11. A 45% crosslinking yield to R17 coat protein was obtained with IU–RNA 2 and polystyrene-filtered emission from a 312-nm, broad-band Spectroline transilluminator. For comparison, only a 20% crosslinking yield was obtained with BrU–RNA 1 and the transilluminator.
12. The diversity of bands generated on excitation at 308 nm is due to photocrosslinking of different amino acids in the protein to at least three different nucleotides which, when substituted with BrdU, result in enhancement of three distinct bands within the gel pattern (*13*).
13. B. J. Hicke, M. C. Willis, T. H. Koch, T. R. Cech, in preparation.
14. RNAs 1, 2, and 3 were prepared by in vitro transcription from synthetic DNA templates by T7 RNA polymerase with [α-^{32}P]CTP [J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **15**, 8783 (1987)]; IUTP was from Sigma. RNA fragments were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). The desired fragment was eluted from the polyacrylamide and ethanol-precipitated in the presence of 0.3 M sodium acetate. R17 bacteriophage was propagated in *Escherichia coli* strain S26, and the coat protein was purified according to the procedure described by J. Carey, P. T. Lowary, and O. C. Uhlenbeck [*Biochemistry* **22**, 4723 (1983)].
15. DNAs 4 and 5 were prepared on a DNA synthesizer (IdU phosphoramidite from Glen Research) and ^{33}P end-labeled. *Oxytricha nova* telomere protein subunits were expressed and purified according to the procedure described by G. Fang, J. T. Gray, and T. R. Cech [*Genes Dev.* **7**, 870 (1993)]. The protein was purified by cation exchange chromatography followed by dialysis against MNG 20/20/20 [20 mM MOPS (pH 7.5), 20 mM NaCl, 20% glycerol] or spin dialysis (Centricon 30 filter, Amicon) into MNG 20/20/20. Protein prepared in this fashion was analyzed by SDS-PAGE and its purity and concentration estimated by comparison to serial dilutions of protein purified according to the procedure described by J. T. Gray, D. W. Celander, C. M. Price, and T. R. Cech [*Cell* **67**, 807 (1991)].
16. A constant, low concentration of ^{32}P-labeled RNA was mixed with a series of coat protein concentrations between 0.06 nM and 1 μM in 10 mM magnesium acetate, 80 mM KCl, bovine serum albumin (BSA) (80 μg/ml), and 100 mM tris-HCl (pH 8.5 at 4°C) (TMK buffer). These were the same conditions used in the crosslinking experiments. After incubation at 4°C for 45 to 60 min, the mixture was filtered through a nitrocellulose filter and the amount of complex retained on the filter determined by liquid scintillation counting.
17. ^{32}P-Labeled RNAs 1 and 2 (5 nM) and R17 coat protein (120 nM) were incubated on ice in 100 mM tris-HCl (pH 8.5 at 4°C), 80 mM KCl, 10 mM magnesium acetate, and BSA (80 μg/ml) for 15 to 25 min before irradiation. These are conditions under which the RNA is fully bound to the coat protein. The RNAs were heated in water to 85°C for 3 min and quick-cooled on ice before use to ensure that the RNAs were in a hairpin conformation [D. R. Groebe and O. C. Uhlenbeck, *Nucleic Acids Res.* **16**, 11725 (1988)]. A Lambda Physik EMG-101 excimer laser was used for irradiations at 308 nm. The output was directed unfocused toward a 4 mm wide by 1 cm path length quartz cuvette containing the RNA-protein complex. The laser was operated in the range of 60 mJ per pulse at 10 Hz; however, only about 25% of the laser beam was incident on the reaction cell. Crosslinked RNA was separated from uncrosslinked RNA by PAGE, and the yields were quantitated with a Phosphor Imager.
18. Supported by the Council for Tobacco Research (M.C.W. and T.H.K.), NIH grants GM-36944 (O.C.U.) and GM-28039 (T.R.C.), an NSF graduate fellowship (B.J.H.), the Howard Hughes Medical Institute, (T.R.C.), and an American Cancer Society professorship (T.R.C.).

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Genetic Relationships Determined by a DNA Heteroduplex Mobility Assay: Analysis of HIV-1 *env* Genes

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The genetic diversity of human immunodeficiency virus (HIV) is a major concern thought to impact on immunologic escape and eventual vaccine efficacy. Here, simple and rapid methods are described for the detection and estimation of genetic divergence between HIV strains on the basis of the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Reliable phylogenetic subtypes were assigned for HIV-1 strains from around the world. Relationships between viruses were closest when derived from the same or epidemiologically linked individuals. When derived from epidemiologically unlinked individuals, the relationships between viruses in a given geographic region correlated with the length of time HIV-1 had been detected in the population and the number of strains initiating widespread infection. Heteroduplex mobility analysis thus provides a tool to expedite epidemiological investigations by assisting in the classification of HIV and is readily applicable to the screening and characterization of other infectious agents and cellular genes.

Human immunodeficiency viruses, like other RNA viruses, exist within their hosts as pools of related genetic variants, often referred to as quasispecies (*1–3*). Within infected individuals the HIV-1 surface envelope (*env*) glycoprotein coding sequences have been found to vary by up to 8%, with an unusually high ratio of nonsynonymous to synonymous mutations, indicative of strong selection for viral surface change, and numerous small in-frame nucleotide deletions and insertions (*4–7*). The highly variable and continuously evolving nature of HIV-1 within individuals accounts for the rapid emergence of viral variants resistant to neutralizing antibodies, cytotoxic T lymphocytes, and antiviral drugs (*8–13*), and contributes to the high level of genetic diversity observed between viral strains identified worldwide. The difficulty in elic-

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