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DNA mismatch repair in *Xenopus* egg extracts: Repair efficiency and DNA repair synthesis for all single base-pair mismatches

(heteroduplex DNA/restriction enzyme/mutagenesis/genetic recombination)

ISABELLE VARLET, MIROSLAV RADMAN, AND PETER BROOKS

Institut Jacques Monod, Centre National de la Recherche Scientifique, 2, Place Jussieu-Tour 43, 75251 Paris Cédex 05, France

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ABSTRACT **Repair of all 12 single base-pair mismatches by *Xenopus* egg extracts was measured by a physical assay with a sequence containing four overlapping restriction sites. The heteroduplex substrates, derivatives of M13 phage DNA, differed in sequence at the mismatch position only and permitted measurement of repair to both strands. The efficiency of repair varied about 4-fold between the most and least effectively repaired mismatches. Repair was most active with C/A and T/C mismatches but the efficiency varied depending on the orientation of the mismatch. Mismatch-specific DNA repair synthesis was also observed but the extent of repair was not always predictive of the extent of synthesis, suggesting the presence of different repair systems or different modes of mismatch recognition.**

Single base mismatches arise by several mechanisms and are corrected by several distinct repair systems in *Escherichia coli* (1–4). Replication errors, whose frequency and specificity depend on the sequence context (5), are corrected by the *mutHLSU* system. The mismatch errors G/T and A/C are corrected more efficiently than purine/purine or pyrimidine/pyrimidine errors, but correction efficiencies are also context-dependent, tuned to complement misincorporation frequencies so that the frequencies of transition and transversion mutations are similar at all base positions (6). *E. coli* also has other specific systems to correct certain replication errors that are poorly repaired by the generalized *mutHLSU* system, such as the A/G → C/G repair by MutY (7–9). T/G mismatches formed upon deamination of 5-methylcytosine in *E. coli* are repaired by a context-specific correction to C/G that is dependent on a gene at the *dcm* locus (10, 11) and on *mutL*, *mutS*, and *polA* (12). A functionally analogous, but context-independent, T/G-specific activity apparently exists in cultured monkey cells (13–15). Mismatches also arise upon formation of recombination intermediates between homologous but nonidentical sequences. In *E. coli*, the *mutHLSU* system aborts recombination intermediates that bear numerous mismatches, thus protecting the genome against interspecific recombinations and against diverse chromosomal rearrangements (16). Mismatch repair systems in eukaryotes, such as in yeast (17, 18) and *Xenopus laevis* (19), may be analogously involved in assuring species divergence, preventing mitotic recombination and other intra- and interchromosomal rearrangements, particularly in view of the presence of large amounts of divergent repeated sequences (16). The degree and local pattern of genome conservation following replication or recombination will reflect the diverse repair efficiencies dependent on mismatch type and on sequence context. In one study with intact monkey cells, comparisons of repair efficiency of different mismatches were confounded because the influence of preferential selection during amplification of the viral DNA repair products was not excluded and because the mismatches were placed in two different sequence contexts (14). An assay that directly scores restitution of restriction sites has been used to evaluate mismatch repair specificity in *E. coli* extracts, with substrates that varied at one or two bases at the fifth position on either side of the mismatch (20). We have used the same approach to study the specificity and mode of action of mismatch repair system(s) in *Xenopus* egg extracts. A repertoire of 12 DNA heteroduplexes with all possible single base-pair mismatches, and differing only at the mismatch site, was used to measure DNA mismatch repair and mismatch-provoked DNA repair synthesis.

MATERIALS AND METHODS

Egg Extract Preparation. Unfertilized-egg collection and extract preparation by differential high-speed centrifugation were as described (19) except that centrifugation was in a Beckman SW50.1 rotor and the supernatant was collected by side-puncture. Protein concentration was about 20 mg/ml. The concentration of dCTP was 44 μM, determined by isotope dilution as described (21).

DNA Preparation and Heteroduplex Construction. Growth of *E. coli* JM105, infection with M13 phage derivatives, and preparation of covalently closed circular double-stranded DNA and single-stranded DNA were as described (22). Oligonucleotides flanked by a *Hin*dIII restriction site at one end and an *Xho* I site at the other were synthesized and cloned into the polylinker of an M13-derived vector, phagescript SK (Stratagene). In addition, the sequence between the sites for *Xho* I and *Kpn* I was replaced with a *Sal* I–*Eco*RI linker. The four resulting derivatives are designated HK7 ("heteroduplex cassette") followed by the letter indicating the restriction site in the cassette (Fig. 1). The modified regions were verified by the dideoxy sequencing method. The 12 heteroduplexes (and homoduplex) were prepared with HK7 replicative form (RF) I that had been linearized by *Bam*HI and with an 8- to 10-fold molar excess of the appropriate HK7 single-stranded DNA. Annealing, incubation with *E. coli* DNA ligase (New England Biolabs), and purification of covalently closed circular DNA were as described (19). Before CsCl centrifugation, heteroduplex preparations were treated with *Cla* I, *Eco*NI, or *Mlu* I as appropriate to minimize DNA sensitive to the repair-diagnostic enzymes. When a heteroduplex preparation contained more than about 10% RF II DNA, the RF I was purified by nitrocellulose filtration (19). For experiments with randomly pre-nicked DNA, a C–A heteroduplex was incubated with DNase I (50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$, 37°C) to yield 20% RF II DNA, extracted with phenol/chloroform, and ethanol-

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Abbreviation: RF, replicative form.

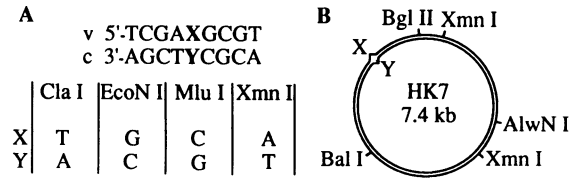

FIG. 1. Heteroduplex substrates for mismatch repair assay. (*A*) Construction of the four HK7 M13 derivatives is described in *Materials and Methods*. The mismatch-neighboring sequences are shown for the viral (v) and complementary (c) strands. X/Y is the position of mismatch. The remaining HK7 sequence is identical for the 12 heteroduplexes. (*B*) The mismatch (<>) is at position 6354. Repair results in restoration of the restriction site; cleavage with *Alw*NI and with either *Cla* I, *Eco*NI, or *Mlu* I generates repair-diagnostic fragments of 4.2 and 3.2 kilobase pairs (kb). Cleavage with *Xmn* I yields a fragment of 2.3 kb and diagnostic fragments of 3.7 and 1.4 kb. Convention for naming base pairs and specifying strand orientation is as follows. X/Y does not specify the strand orientation and does not specify whether the bases are mismatched or complementary. For X–Y (a mismatched pair) and X·Y (a complementary pair), X is in the viral (v) strand and Y is in the complementary (c) strand.

precipitated. Control DNA that was processed identically except without DNase I was <5% RF II.

Mismatch Repair Assay. Incubations at 22°C for 45 min (except as indicated) included 20 μM heteroduplex DNA and 70% (vol/vol) egg extract. Buffers and salts from the extract were supplemented so that the final reaction mixtures contained 27 mM potassium Hepes (pH 7.4), 80 mM potassium glutamate, 10 mM magnesium acetate, 25 mM potassium acetate, 1.3 mM dithiothreitol, each of the four dNTPs at 50 μM (including 30 μM from the extract, as estimated from the determination of dCTP), 4 mM ATP, 1.75% (wt/vol) sucrose, and bovine serum albumin at 200 μg/ml. The reactions were stopped and DNA was extracted as described (19). After linearization with *Alw*NI, the DNA was probed with the appropriate restriction enzyme for mismatch repair (Fig. 1) and the diagnostic fragments were separated by agarose gel electrophoresis in 40 mM Tris acetate, pH 8.5/1 mM EDTA with ethidium bromide at 0.5 μg/ml. After electrophoresis, DNA was transferred to Hybond-N+ (Amersham) under denaturing conditions as recommended by the supplier. Hybridization with ^{32}P-labeled M13mp18 and washing were as described (23, 24). Autoradiograms, exposed at room temperature without intensifying screens, were scanned with a Chromoscan3 densitometer (Joyce–Loebl) and integration was done with the data analysis programs of the Chromoscan. Radioactivity in some blots was also determined with a two-dimensional detector (Autograph, Saxon Micro, Newmarket, U.K.). Quantitative digestion by *Alw*NI, *Mlu* I, *Eco*NI, and *Cla* I was verified by the observation in ethidium bromide-stained gels of complete digestion (>95%, data not shown) of λ phage DNA that was added to each restriction digestion. The *Xmn* I reactions included fragments of HK7-X DNA prepared by prior cleavage with *Bal* I, *Alw*NI, and *Bgl* II; the autoradiograms showed quantitative digestion of these fragments by *Xmn* I (>95%, data not shown). In parallel experiments, the four HK7 RF I DNAs incubated with the extract under repair assay conditions were completely digested by the appropriate enzymes, thus validating the internal control of addition of λ DNA or the HK7-X fragments immediately before incubation with the restriction endonucleases.

DNA Repair Synthesis. Reactions were comparable to those for repair (described above), except that the DNA concentration was reduced by a factor of ≈5, exogenous dNTPs were not included, and [α-^{32}P]dCTP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) was added. The reactions were stopped at the indicated times with EDTA (30 mM final) and radioactivity incorporated was measured by perchloric acid precipitation, filtration through glass-fiber filters (Whatman GF/C), and scintillation counting.

RESULTS

Assay for Repair of the 12 Mismatches in an Invariant Sequence. The repair assay uses mismatch-containing restriction sites that are resistant to cleavage by the restriction enzymes (25). When a mismatch is converted to a complementary base pair by incubation with the extract, subsequent cleavage with the repair-diagnostic restriction enzyme and cleavage at one or more other sites produce fragments that are diagnostic for repair. Repair to both strands can be assayed using overlapping restriction sites (26); we designed a sequence to permit comparison of repair efficiency of the eight different single base mismatches with sequence context effects completely eliminated (Fig. 1). Because the only sequence difference was at the variant position, the four heteromismatches (T/G, C/A, A/G, and C/T) could be placed in either orientation. Therefore, the 24 different repair events, repair to each strand of each of the 12 mismatches, could be scored by restoration of one of the four overlapping restriction enzyme sites.

Blotting and hybridization with radiolabeled DNA was chosen as the method of quantification to assure detection of all repair events. The sensitivity of this method revealed that occasionally the diagnostic enzymes cleaved the heteroduplex preparations at levels not detectable by ethidium bromide staining. Values for repair were appropriately corrected. Typical results are shown in Fig. 2. In the absence of the extract, heteroduplexes T–C and G–G (see Fig. 1 legend) were not cleaved by the diagnostic enzymes (<0.1%; Fig. 2, lanes −). After incubation with the extract, different levels of

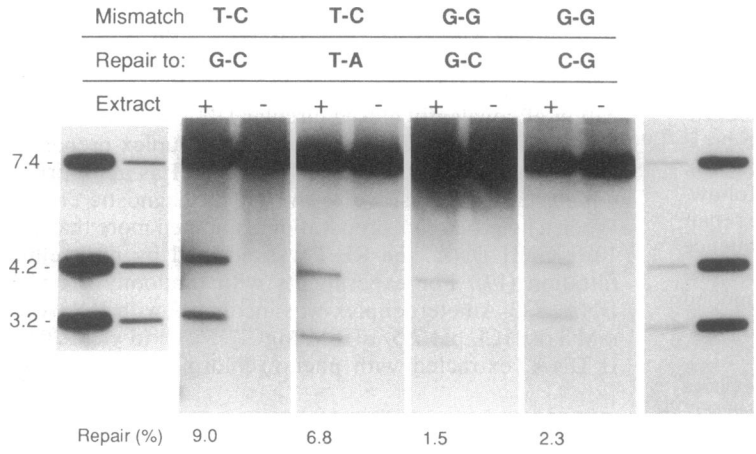

FIG. 2. Quantification of mismatch repair. Reactions with heteroduplex DNA containing the indicated mismatches, validation of complete digestion by the restriction enzymes, and measurement of repair were as described in *Materials and Methods*. Pairs of lanes from different experiments are shown. Densitometry calibration with several autoradiogram exposures was with linear 7.4-kb HK7 DNA and the 4.2- and 3.2-kb fragments from RF I homoduplex DNA (two leftmost lanes and two rightmost lanes) and by normalization of the integrals of the linear 7.4-kb band of the autoradiograms to those of the photographic negative. The exposures shown were used to quantify the repair-diagnostic fragments (4.2 and 3.2 kb) and much briefer exposures to quantify the 7.4-kb band.

repair were observed for the different mismatches (lanes +). Diagnostic bands were always produced in approximately equivalent molar quantities; for repair scored by *Mlu* I, *Eco*NI, and *Cla* I, this enabled duplicate determinations of the extent of repair based on the individual quantification of each diagnostic band. In addition, the repair of each of the 12 heteroduplexes was measured in two to four separate experiments. The radioactivity in some blots was also measured directly by counting in a two-dimensional radioactivity detector; these measurements agreed with values for repair extent determined by densitometry (data not shown). Two types of experiments showed that variable amounts of RF II in the heteroduplex preparations did not influence the results (data not shown). First, the extracts repaired partially pre-nicked heteroduplex DNA (20% RF II; see *Materials and Methods*) to the same extent as control DNA. Second, no significant difference was observed between results obtained with heteroduplex preparations before and after the nitrocellulose filtration procedure that enriches for closed circles.

Efficiency of Repair Varies for Different Mismatches. The 12 heteroduplexes were individually incubated with the extract and then probed with the appropriate restriction enzymes to measure repair to each base in the mismatch. As shown in Fig. 3, the heteroduplexes were repaired with efficiencies that varied over 4-fold between the most efficiently (C–A) and least efficiently (G–T, G–G) repaired. This variation demonstrates that the extract contains activities that are responding specifically to the mismatches.

The differences are dependent on the mismatches themselves, as there is no systematic strand bias and none of the four sequences was favored. Reversal of the orientation of the mismatch did not affect the efficiency of repair of A–G compared with G–A. In contrast, although repair of C–A to C·G and repair of A–C to G·C were not different, repair of C–A to T·A was about 4 times greater than repair of A–C to A·T. In addition, T–G was repaired about 4 times better than G–T, and the data suggest that T–C was repaired more efficiently than was C–T. Depending on the mechanism of mismatch recognition, inversion of mismatch orientation could be equivalent to positioning the mismatch in a different sequence context. Therefore, the dependence of repair efficiency on orientation may reflect differences in heteroduplex structure induced by these mismatches or differences in the efficiency of recognition of certain mismatches dependent on the local sequence environment (see *Discussion*).

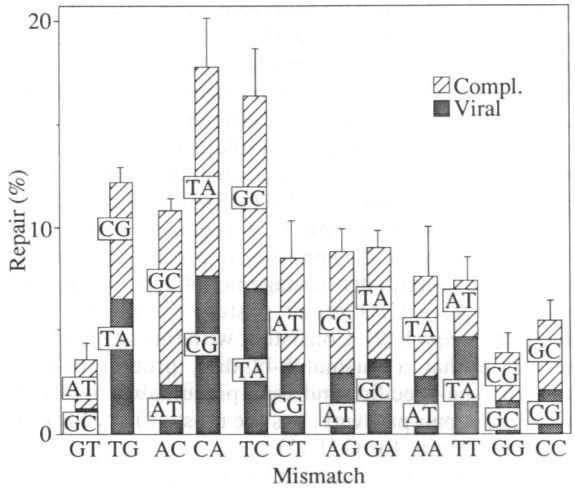

FIG. 3. Specificity of repair. The height of each bar is the sum of repair to the base in the viral strand (stippled) and to the base in the complementary strand (hatched). The error bars represent a standard deviation of the mean of repair for both strands. In control assays in the absence of extract, in most experiments, heteroduplexes were not cleaved by the diagnostic enzymes (<0.1%). Controls that exceeded 15% of the extract-dependent determination were as follows (values are % DNA in diagnostic fragments from duplicate experiments): G–A to G·C (1.1, 0.7), G–T to A·T (0.7, 1.2), C–T to A·T (2.4, 2.7), A–C to A·T (1.5, 1.9), T–T to A·T (1.8, 0.9).

Mismatch-Stimulated DNA Repair Synthesis. Repair efficiency that is dependent on mismatch type shows that the repair activity is specifically responding to the mismatches but does not rule out the possibility that repair of the least effectively repaired mismatches is due to random strand replacement synthesis. Therefore, we measured mismatch-stimulated DNA synthesis. The kinetics of repair, as assayed with the mismatch C–A (Fig. 4*A*), were similar to the kinetics of mismatch-provoked DNA synthesis (Fig. 4 *B* and *D*), thus suggesting that the two activities are related. The extent of synthesis induced by the heteroduplexes exceeded that in homoduplex DNA by a factor of 2–7 (Fig. 4 *B–D*). Because this range is comparable to the 4-fold variation in repair efficiency (Fig. 3) and because mismatch-specific synthesis did occur even for less efficiently repaired mismatches (C–C, Fig. 4*B*, and G–T, Fig. 4*D*), we conclude that repair of these mismatches was due to a mismatch-dependent process. In addition, these experiments show that measurement of DNA repair synthesis by acid-precipitable incorporation of radiolabeled dNTP can serve as a facile assay for mismatch repair in the *Xenopus* egg extracts.

The most efficiently repaired mismatches, C–A and T–C, induced the most incorporation (Fig. 4 *B* and *D*) whereas C–C, a less efficiently repaired mismatch, induced the lowest amount of synthesis (Fig. 4*B*). The extent of repair, however, did not necessarily predict the extent of mismatch-dependent synthesis. For example, whereas a difference in repair efficiency between the two orientations was observed with T/G and A/C and was suggested with T/C (Fig. 3), no orientation-dependent difference in repair synthesis was observed for these three mismatches (Fig. 4). We infer that different mismatches can induce variant amounts of repair synthesis per mismatch repaired. Different levels of synthesis were not due to variable amounts of RF II in the heteroduplex preparations: pre-nicked DNA did not generate increased levels of incorporation (data not shown). Lower levels of incorporation were not due to an inhibitor: when the extract was incubated with two heteroduplexes, one that induced a high

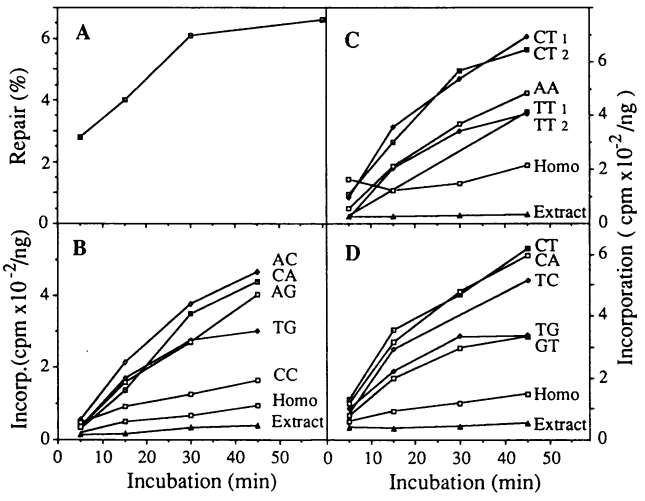

FIG. 4. Kinetics of repair and repair synthesis. (*A*) Kinetics of repair of C–A to C·G. (*B–D*) Kinetics of incorporation of [α-^{32}P]dCTP (cpm/fmol of total dCTP: *B* and *C*, 7.6; *D*, 6.4) either with no added DNA ("Extract") or with homoduplex or heteroduplex DNA. Of the 12 mismatches, only G–A and G–G were not tested. Each panel represents a different experiment; in *C*, 1 and 2 indicate duplicate incubations of two different heteroduplex preparations.

level of synthesis and the other a low level, incorporation remained at the higher level (data not shown).

It appears that C–A or A–C induced more mismatch-specific synthesis than did T–G or G–T (Fig. 4 *B* and *D*) after 45 min of incubation. These comparisons, however, are potentially compromised by inherent errors in corrections for nonspecific synthesis and in substrate normalization. Therefore, we examined intramolecular DNA synthesis, an analysis that excludes both of these sources of error. In agreement with previous results (19), mismatches in the HK7 substrate provoked mismatch-localized synthesis (Fig. 5). If fragments A or C represent primarily nonspecific synthesis, then the amount of mismatch-localized synthesis for C–A (Fig. 5*C*) is greater than that for G–T (Fig. 5*B*).* Thus, taken together, the results shown in Figs. 4 and 5 indicate that G–T induced less synthesis than C–A. This concurs with the finding that G–T was less efficiently repaired than C–A (Fig. 3).

DISCUSSION

We have previously shown that DNA repair synthesis associated with mismatch repair in *Xenopus* egg extracts is localized to the mismatch-bearing region; we concluded that the repair was due to a genuine mismatch repair system and not simply to restoration of the restriction sites by randomly occurring nick–translation events (19). We show here that the activity discriminates between different mismatches, resulting in different efficiencies of repair and different extents of mismatch-stimulated DNA repair synthesis. Such discrimination is typical of *in vivo* mismatch repair in other organisms and suggests that the *Xenopus* extract contains repair systems that are active *in vivo* in response to mispaired bases.

The specificity of repair by the extract is similar in some respects to the specificities reported for mismatch repair in lower organisms. The transition mismatches, C/A and T/G, are consistently repaired more efficiently than the transversion mismatches in *E. coli* (5, 27), *Streptococcus pneumoniae* (4), and yeast (17, 28). We have found, however, that while C/A was well repaired (at least to one strand), repair of T/G was variable, with T–G repaired with relatively moderate efficiency and G–T poorly repaired. In addition, we have not seen the biased, but context-independent, repair of T/G to C/G that has been reported for monkey cells (13, 14). This bias has been interpreted as reflecting an activity that protects the DNA from mutations arising from deamination of 5-methylcytosine (13, 14). The oocyte, however, is primed to sustain twelve 30-min doublings of its 3×10^9-base-pair genome (29), so that mismatch repair of DNA replication errors may predominate. Activities that correct mismatches arising independently of replication, such as deamination of 5-methylcytosine, may thus be masked just as *dcm*-dependent VSP (very short patch) repair in *E. coli* cannot readily be observed when the *mutHLSU* system is active (11). Alternatively, although there is no evidence for base-specific repair systems in *Xenopus*, analogy with prokaryotic systems and results from other eukaryotic studies suggest other possible explanations. (*i*) An activity that specifically repairs T/G to C/G might be inhibited during replication to prevent strand-ignorant correction. If this inhibition involved a binding protein specific for T/G, such as that found in extracts of human cells (30, 31), such binding might also then impede repair by the generalized system. Perhaps this latter

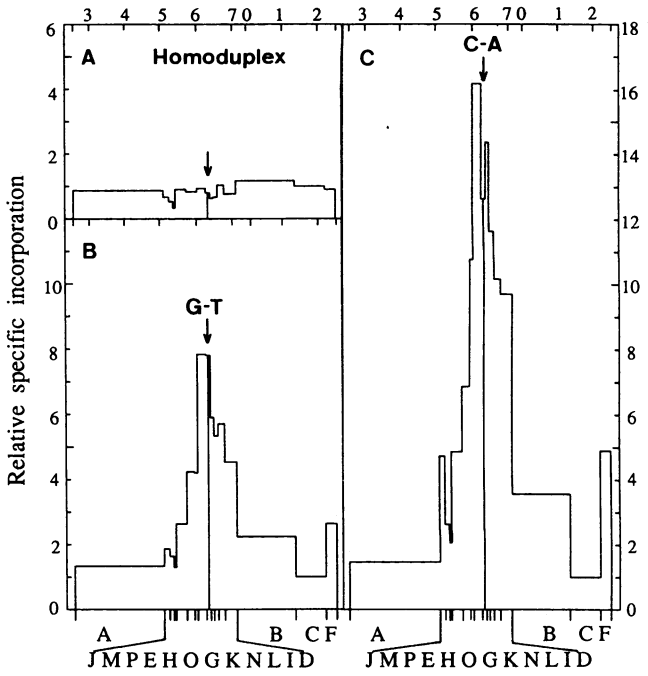

FIG. 5. Mismatch-localized synthesis. Reaction mixtures including [α-^{32}P]dATP and [α-^{32}P]dCTP (8.6 cpm/fmol of total dATP or dCTP) and the substrates as indicated at 3 μM were incubated for 45 min. After postreaction digestion with *Hae* III, fragments were resolved by 8% polyacrylamide gel electrophoresis, and autoradiograms of the dried gel were quantified by densitometry. Analysis was as described (19); fragments E and F comigrated and so half of their total density was arbitrarily attributed to each. Map positions (kb) are indicated on the top axis; arrow and corresponding vertical line indicate mismatch position. Normalization shown is with fragment C; normalization with fragment A yielded a similar pattern except that the maximum relative specific incorporation values for the peak fragments for G–T (*B*) and C–A (*C*) were about 6 and 12, respectively.

undesirable effect would be avoided when strand discrimination signals, such as nicks, are present. (*ii*) In addition, to counteract the potentially mutagenic C/G-biased repair of newly replicated DNA, 5-methylcytosine-containing organisms may have evolved replicases that minimize the frequency of T/G misincorporation errors (specifically, G opposite template T). Then, a less efficient correction of T/G, as we have observed, would complement the lower replication error frequency. An analogous pair of activities in *E. coli* apparently compensates for the inefficient repair of G/A by the *mutHLSU* system. MutT, a replicase protein, deters misincorporation of G opposite template A (32) and so counters the potentially mutagenic effects of the strand-ignorant A/G → C/G repair system (7, 8, 33), which is dependent on *mutY* (9). Consistent with this suggestion is the observation that a mismatch-binding protein that is not mismatch type-specific, and thus presumably involved in a generalized repair pathway, has the most affinity for A/C but little or no affinity for G/T (31).

The differences in repair efficiency of T–G vs. G–T, of T–C vs. C–T, and of C–A vs. A–C (Fig. 3) may reflect differences due to sequence context. The heteroduplex substrates were designed such that each of the heteromismatches could be inverted without requiring any other sequence changes. In this respect, however, inversion of a single base mismatch may be considered as the equivalent of placing the same mismatch in a different sequence. Reversal of mismatch orientation can affect thermal stability and helix melting enthalpy (34, 35). In addition, as shown in Table 1, in a sequence with significant similarities to the HK7 sequence,

*The intramolecular synthesis distributions impose an upper limit on the specific incorporation in molecules repaired by a mismatch-specific system and thus, together with the determination of the fraction repaired (Fig. 3), a minimum estimate of the fraction specifically repaired can be determined (19). For G–T, this conservative estimate is about 50%, and for C–A, >90%.

Table 1. Comparison of chemical reactivity and mismatch repair efficiency

	Reactivity or repair of mismatch X–Y					
	G–T	T–G	C–A	A–C	T–C	C–T
Reactivity						
Osmium tetroxide	+*	+			+	−
Hydroxylamine			+	−	−	+
Repair, %	4.5	13.4	18.7	9.5	17.2	8.9

The chemical reactivity data (36) and the repair data (Fig. 3) were obtained using the sequences shown aligned below.

Sequence from ref. 36 5′-C C G A X G T G G
HK7 sequence (viral strand) 5′-T C G A X G C G T

Sequence identities are indicated by vertical lines. X is the mismatch position. The complementary strands (containing the mismatch base Y) are not shown.

*With G–T, the thymine 3′ to the mispaired thymine was also reactive with osmium tetroxide.

including identity of the bases flanking the mismatch, the reactivity of the pyrimidines in the mispairs C/A and T/C with osmium tetroxide or with hydroxylamine depended on orientation (36). Moreover, although the thymines in G–T or in T–G were both reactive with osmium tetroxide, only the G–T mispair enabled reactivity of the flanking thymine. These differences in reactivity with the chemical probes are paralleled by the orientation-dependent repair of these mismatches in the *Xenopus* extracts (Fig. 3 and Table 1). Mismatch repair efficiency influenced by sequence context has also been documented in *E. coli* and *S. pneumoniae* (2, 4, 5, 37). Indeed, in some cases, transversion mismatches were as efficiently repaired as some transition mismatches (4, 5, 20).

Unequal repair was observed with only the A–C mismatch. For the remaining mismatches, despite the differences in total correction efficiency to both strands, repair was distributed about equally on both strands, as occurs in *E. coli* in the absence of a strand discrimination signal (38). This is in striking contrast to the results in monkey cells, where unequal repair was observed for most of the mismatches, including biases exceeding 10-fold for G/G and more than 20-fold for T/G (14). The T/G → C/G bias was interpreted as due to a T/G-specific repair system (14). It is possible that the other biases in monkey cells and the bias observed here for A–C repair are also due to repair systems or proteins specific for particular mismatches and perhaps only when these mispairs are located in certain sequence contexts. Indeed, the *micA*/*mutY* system apparently removes the A in A/C as well as in A/G mismatches (8).

We have previously shown that the *Xenopus* egg extract acts on heteroduplex DNA to produce repaired molecules with mismatch-localized synthesis and molecules that have undergone mismatch-provoked and mismatch-localized DNA synthesis but remain unrepaired (19). This seemingly unproductive synthesis may be mismatch type-dependent, as some mismatches induced more repair synthesis per repair event than others (e.g., G–T vs. T–G, Figs. 3 and 4). Therefore, multiple levels of mismatch recognition (39), or factors other than those that bind the mismatches and that enable the initiation of DNA repair synthesis, may be involved in determining the efficiency of repair. This situation may be functionally comparable to the *E. coli mutHLSU* system, where the repair efficiency of different mismatches does not always correlate with MutS binding affinity (20).

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