be used is restricted mainly by the size of the microscope field of view (which can be increased using smaller magnification objectives at the expense of visibility of fluorescent signals) and by the difficulty to assess whether two apparently distant and aligned signals belong to the same fiber or not, setting a practical distance limit of a few hundred kilobases [400 kb, 40× objective (*21*)]. Detection of the underlying DNA fiber with another fluorochrome (*16*) does not resolve this ambiguity when the density of combed fibers is too high, because of the possible confusion between close fibers (*21*). Hybridization of several probes together, detected with multicolor fluorescent systems, would decrease the number of hybridizations and the scanning time necessary for the construction of large high-resolution physical maps, contrary to optical mapping techniques that require one experiment per clone.

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27. This yield of about 50 to 100 pairs of hybridized cosmids is in agreement with the rough estimate of DNA density of, at most, a few hundred combed genomes per slide (*14*). The total number of genomes as estimated, also including the incomplete and isolated signals, is indeed much closer to the one estimated from combing density, thus showing a satisfactory hybridization efficiency (R. Ekong and X. Michalet, data not shown; J. Herrick *et al.*, in preparation).
28. In both experiments, comparison of measurements done on different slides (slides prepared on different days but from the same DNA solution or from different DNA agarose blocks using either the same or different batches of silanized cover slips) gave similar results.
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36. X.M. benefited from a Pasteur-Weizmann grant, S.R. from a Wellcome Trust grant, and M.V.S. from support by the Dutch Organisation for Scientific Research. We thank A. Chiffaudel for technical help in the development of the combing apparatus, J. Sampson and P. Harris for providing us with TSC2 probes and patient cell lines, and J. Nahmias for unpublished data on the TSC1 contig. Supported in part by the Association Française contre les Myopathies. Dedicated to A. Ullmann for constant support throughout this project.

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Conditional Mutator Phenotypes in hMSH2-Deficient Tumor Cell Lines

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Two human tumor cell lines that are deficient in the mismatch repair protein hMSH2 show little or no increase in mutation rate relative to that of a mismatch repair–proficient cell line when the cells are maintained in culture conditions allowing rapid growth. However, mutations accumulate at a high rate in these cells when they are maintained at high density. Thus the mutator phenotype of some mismatch repair–deficient cell lines is conditional and strongly depends on growth conditions. These observations have implications for tumor development because they suggest that mutations may accumulate in tumor cells when growth is limited.

The autosomal dominant syndrome of hereditary nonpolyposis colon cancer (HNPCC) is characterized by early onset of colon tumors as well as cancers of the endometrium, stomach, upper urinary tract, small intestine, and ovary. Mutations in two human homologs of the *Escherichia coli* mismatch repair genes *MutS* and *MutL* (*hMSH2* and *hMLH1*) are found in the great majority of HNPCC patients (*1*). Less frequent germline mutations of another *MutL* homolog (*hPMS2*) are also associated with this disease (*2*). HNPCC patients inherit a mutant allele of a mismatch repair gene (*3*), and the second wild-type allele is mutated or lost as an early event in tumor development (*3*, *4*). This second event renders cells mismatch repair–deficient (*5*, *6*), presumably leading to a mutator phenotype that drives the accumulation of mutations required for tumor development (*7*). Two other genes encoding homologs of the *E. coli MutS* gene (*hMSH3* and *hMSH6*) appear to be involved in the repair of some types of DNA replication errors or damage (*8*). A variety of in vitro assays indicate that heterodimers formed between these proteins and hMSH2 exhibit considerable specificity in the types of errors they recognize and bind. Thus, hMSH2 may play a central role in recognition of DNA replication errors while hMSH3 and hMSH6 modify the specificity of this recognition (*8*).

To examine the consequences of hMSH2 deficiency in human tumor cells, we measured mutation rates in two tumor cell lines with *hMSH2* mutations: SK-UT-1, which was derived from a uterine tumor; and 2774, which originated from an ovarian tumor (*6*, *9*–*11*). SK-UT-1 has a 2–base pair (bp) deletion in exon 10 of the *hMSH2* coding sequence that results in a truncation (*9*); and 2774 has a base substitution in exon 14, resulting in a missense mutation (Arg → Pro) (*10*). SK-UT-1 has no detectable hMSH2 protein, whereas 2774 retains a full-length mutant protein (*12*). The levels of hMLH1 and hPMS2 in these cell lines are similar to those in repair-proficient cell lines. Surprisingly, mutation rates of growing 2774 and SK-UT-1 cells at the X-linked locus encoding the purine salvage enzyme hypoxanthine guanine phosphoribosyl transferase (*HPRT*) (Table 1) were lower than that measured for the mismatch repair–proficient SV40-transformed fibroblast line MRC-5 (rate = 1.4×10^{-7} mutations per cell per generation). This contrasts with the 130- to 190-fold increase in mutation rate found in tumor cell lines deficient in other mismatch repair components (Table 2). Other laboratories have reported difficulties in isolating *HPRT* mutants from hMSH2-deficient cell lines (*13*), although increased mutation rates have been measured in the hMSH2-deficient LoVo cell line (*14*, *15*). To test the possibility that the low *HPRT* mutation rate in the hMSH2-deficient cells is due to the presence of multiple active X chromosomes (*16*), we measured the rate of mutation to ouabain resistance (Oua^R). Because these mutations act dominantly, they are not obscured in polyploid cells (*17*). However, even at this locus there was no change in mutation rate for cell line 2774 relative to that in mismatch repair–proficient MRC-5, whereas that in SK-UT-1 was elevated 7.1-fold (*18*). Thus, these data indicate that the two hMSH2-deficient tumor cell lines develop a weaker mutator phenotype than lines deficient in other mismatch repair genes.

To determine whether mutation rates increased in these hMSH2-deficient cells under less optimal culture conditions, we examined *HPRT* and Oua^R mutant frequencies in 2774 and SK-UT-1 cells that had been maintained for 2 weeks in a high-density growth-limited state. For 2774 cells, the *HPRT* mutant frequency of replicas maintained at high density was 7900-fold higher and the frequency of Oua^R mutants was >67-fold higher relative to those of replica cultures kept under optimal growth conditions (Table 1). SK-UT-1 cells main-

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Table 1. Accumulation of mutations in tumor cell lines maintained at high density.

Locus	2774				SK-UT-1	
	HPRT		*Oua*^{R}		*HPRT*	
Culture type*	Growing	High density	Growing	High density	Growing	High density
Final cell number (mean)	7.9×10^6	7.6×10^5	2.4×10^6	7.5×10^5	5.1×10^6	3.1×10^6
Replicas	24	24	17	24	20	12
Replicas with mutants	1	24	0	11	7	11
Mutant range per replica	0–1	4–65	0	0–4	0–12	0–29
Mutant frequency (mean)	7.8×10^{-9}	6.2×10^{-5}	$<3.9 \times 10^{-8}$	2.6×10^{-6}	3.5×10^{-7}	1.2×10^{-5}
Mutation rate†	9.3×10^{-9}	ND	$<4.4 \times 10^{-8}$	ND	1.0×10^{-7}	ND

*Both growing and high-density cultures originated from the same pool of replica cultures initiated with 100 cells per 35-mm tissue culture well. At 2 weeks after inoculation of the replicas, half of the cultures were treated with trypsin and transferred to 100-mm dishes. These growing cultures were harvested for assay of *HPRT* or *Oua*^{R} mutants 5 days later. Cells in high-density replicas were treated with trypsin and spread on the same 35-mm well to limit growth. These cultures were harvested for assay of *HPRT* or *Oua*^{R} mutants 2 weeks later. The viability of cells in the cultures maintained under these restrictive growth conditions was slightly lower, 50% for 2774 and 21% for SK-UT-1 as compared with 64% and 67%, respectively, for the two lines maintained under optimal growth conditions. Mutant frequencies were corrected for plating efficiency. †The mutation rate was calculated by the method of the mean. ND, not determined. We did not calculate the mutation rate of high-density cultures because the mutations occurred only after growth was limited.

tained at high density showed a 34-fold increase in frequency of *HPRT* mutants.

To determine the nature of the mutations in the cells maintained in restrictive conditions, we picked independent colonies from each of the replica cultures and generated *HPRT* cDNAs by reverse transcription (*19*). The mutant cDNAs were amplified and sequenced. Over half of the mutations (7 of 13) identified in 2774 were frame shifts at mono- or dinucleotide runs (Table 3). The remaining *HPRT* mutations were transitions, transversions, and a frame shift outside the runs. In SK-UT-1 cells, six of seven *HPRT* mutations were frame shifts. This distinctive pattern of frame shifts is also evident in other log-growing mismatch repair–deficient cell lines (*14*, *20*).

We next examined microsatellites in single cell clones isolated from line 2774 to determine whether they exhibited a similar conditional instability (*21*). Four loci were amplified with DNA purified from 20 independent subclones isolated from growing 2774 cells. No novel microsatellite alleles were detected at these loci (frequency <1.7%). In contrast, novel alleles were detected in 36% of the subclones isolated from 2774 cultures maintained at high density. In some subclones there were losses or

Table 2. *HPRT* mutation rates in growing and high-density cultures of mismatch repair–deficient tumor cells.

Culture type*	DLD-1		HEC-1-A		
	Growing	High density	Growing	High density	Replica sample control†
Number of cells per replica (mean)	5.6×10^5	7.6×10^5	3.1×10^5	7.5×10^5	3.8×10^5
HPRT mutant colonies (left column) and number of replica cultures (right columns)					
$n = 0$	0	0	6	0	0
2	1	0	4	0	0
3–4	0	0	3	0	0
5–8	3	1	0	0	0
9–16	5	7	2	0	3
17–32	5	3	7	0	9
33–64	4	5	0	1	0
65–128	3	3	1	12	0
129–256	0	0	0	9	0
257–512	1	2	1	1	0
513–1024	1	2	0	1	0
>1024	1	1	0	0	0
Mutants per replica					
Range	2–976	7–1000	0–370	53–925	15–22
Mean	88.5	150.3	28	159	19
Mutant frequency	1.6×10^{-4}	2.0×10^{-4}	9.0×10^{-5}	2.1×10^{-4}	5×10^{-5}
Mutation rate‡	2.7×10^{-5}	ND	1.8×10^{-5}	ND	ND

*Both growing and high-density cultures originated from the same pool of replica cultures initiated with 100 cells per 35-mm tissue culture well. At 2 weeks after inoculation of the replicas, 24 of the cultures were treated with trypsin and plated on 6-thioguanine (6-tg) medium for determination of the number of *HPRT* mutants. The remaining 24 replica cultures were left for an additional 2 weeks before being plated in 6-tg medium. The plating efficiency of these cultures remained at 80 to 90% after this treatment. The change in the number of mutants per replica culture between growing and high-density cultures was tested by an *F* test for equal variance and found to be highly significant ($P < 0.0001$). †In the replica sample control, a single replica culture was allowed to grow to a large cell number. This culture was then sampled 12 times (3.8×10^5 cells per sample) for plating on 6-tg medium. This value shows the range in mutant number obtained from repeated sampling of the same culture. ‡The mutation rate was calculated by the method of the mean. ND, not determined (see Table 1).

gains of multiple repeat units (Fig. 1). This pattern is consistent with a previous report of increased microsatellite instability in subclones of 2774 maintained for a prolonged period (*10*). We also examined microsatellite stability in *HPRT* mutants isolated from cultures maintained at high density and again observed dramatic alterations in a high proportion (46%) of subclones (Fig. 1).

Our attempts to determine whether other mismatch repair–deficient cell lines exhibit a similar response were complicated by the high frequency of mutants accumulating in replica cultures before they reached high density. As a result, replica cultures of the hPMS2-deficient endometrial carcinoma cell line HEC-1-A maintained at high density showed only a small (2.3-fold) increase in *HPRT* mutant frequency relative to that of growing replicas (Table 2). More interesting was the loss of variation in the number of mutants in the high-density replica cultures (Table 2). The change in variation observed in high-density cultures was highly significant ($P < 0.0001$), and the variation in mutant number per high-density replica was similar to that found when a single culture of HEC-1-A cells was repeatedly sampled (Table 2). High-density cultures of the colon cancer tumor cell line DLD-1, which is deficient in hMSH6 and has a missense mutation of DNA polymerase δ (*22*), did not show a similar effect (Table 2). Because mutation is a random process, the number of mutants is normally highly variable, depending on when the mutation occurs during the outgrowth of a replica culture (*11*, *17*). The loss of variability in HEC-1-A replicas maintained at high density indicates that an inductive process leads to the accumulation of mutants under such conditions. Alternatively, the much lengthened time for the expression of the HPRT deficiency in the high-density cultures may have improved the recovery of mutants. However, the fact that DLD-1 did not accumulate mutants at high density over the same length of time argues against the latter hypothesis.

Although we have clearly seen the conditional mutator phenotype in two hMSH2-deficient lines, it is not known whether this property is restricted to a subset of mismatch repair–deficient cells or occurs in a wide variety of tumor cells. The extraordinary increase in mutant frequency in 2774 cells could be the result of the stable expression of the mutant hMSH2 protein. This protein may retain some residual activity (*6*) that suppresses the mutation rate under growing conditions. Under conditions of stress, this activity may be saturated by an elevated level of DNA replication errors or damage. The increase in mutant frequency in SK-UT-1 cells maintained at high density relative to that in growing cells was not as dramatic. In part this can be attributed to the higher (nearly 50-fold) baseline mutant frequency of the growing cells. HEC-1-A, which is deficient in hPMS2, also continues to accumulate *HPRT* mutants when maintained under restrictive conditions. However, the mutator phenotype in these cells is not strictly conditional because HEC-1-A has a high rate of mutation under optimal growth conditions.

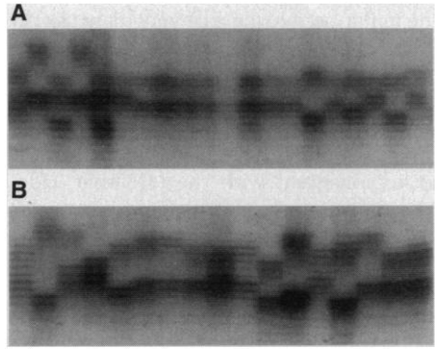

Fig. 1. Microsatellite instability in single cell clones of 2774 cultures maintained at high density without selection (**A**) and in *HPRT* mutant strains selected from high-density replicas (**B**). The dinucleotide repeat locus *D10S197* was assayed. The left lane of each panel contains microsatellites amplified from DNA purified from uncloned, growing 2774 cells. Single cell clones obtained from growing cultures of 2774 had no alterations at this locus (*21*).

The frame shifts in hMSH2-deficient cells at high density indicate that the elevated mutant frequency is the consequence of DNA replication occurring in the absence of mismatch repair. This DNA synthesis could occur in a small subpopulation of cells that continue to grow in spite of the restrictive culture conditions. Alternatively, the accumulation of mutations may be the product of an error-prone DNA repair pathway induced by the suboptimal culture conditions. If the first hypothesis is correct,

Table 3. *HPRT* mutations in 2774 and SK-UT-1 cells maintained at high density.

Mutant	Exon	Position*	Mutation	Target sequence†	Alteration
2774					
DE5.1	1	2	T → C	CGTTA **T** GGCGA	Met → Thr
DE20.1.1	1	27	G → T	GTCGT **G** gtgag	Altered splice
DE3.2		*1706*	T → C	CGTGg *t* gagca	Altered splice
DE20.1.5	2	101	+T	GGAAA GGGTG	Frame shift
DE2.1	2	133	A → G	TGGAC **A** GGACT	Arg → Gly
DE14.2	3	196	−TG	CCCTC **TGTGTG** CTCAA	Frame shift
DE11.2	3	207	+G	CTCAA **GGGGGG** CTATA	Frame shift
DE19.1					
DE20.1.4					
DE9.1	6	484	A → C	TCGCA **A** GCTTG	Ser → Arg
DE16.1	7	496	−A	TGGTG **AAAA** GGACC	Frame shift
DE18.2					
DE20.1	9	636	−A	ACTGG **AAAA** GCAAAA	Frame shift
SK-UT-1					
DE9.2	3	207	+G	CTCAA **GGGGGG** CTATA	Frame shift
DE8					
DE6					
DE4					
DE1	7	496	−A	TGGTG **AAAA** GGACC	Frame shift
DE9.1	8	602	A → G	CAGGG **A** TTTGA	Asp → Gly

*Position of the mutation in the *HPRT* cDNA sequence, with nucleotide 1 being the A in the ATG initiation codon. In DE3.2, the mutation occurred at the slice donor just inside intron 1. The position of this mutation (in italics) represents the nucleotide number in the genomic sequence (*19*). †Nucleotides in upper case represent those in the *HPRT* coding sequence. Lower-case nucleotides are from introns. The nucleotide mutated in these 6-tg–resistant strains is indicated in bold. In the case of the frame shifts in runs of nucleotides, the entire repeat is in bold. The position of the frame shift that did not fall in a run is indicated by a gap in the sequence.

the mutation rate must be very high, as only a small proportion of cells in these cultures (7.5%) have an S-phase DNA content. The high level of microsatellite instability in subclones isolated from high-density cultures and the *HPRT* mutant strains appears to be consistent with the idea that widespread catastrophic DNA synthesis occurs in these cells. Thus, the conditional mutator phenotype may reflect the loss of a checkpoint that prevents cells from entering the S phase when environmental conditions are not optimal, which is not unlike the checkpoint that arrests cells exposed to hypoxia (*23*). With respect to the possibility of error-prone repair in cells maintained at high density, it was recently reported that transient exposure of a mouse tumor cell line to hypoxia modestly increased the mutant frequency of a target gene (*24*). Mismatch repair has thus far been primarily associated with correction of DNA replication errors in growing cells, but may have another function or functions outside the S phase (*25*).

Our observations raise the possibility that mutations in some cells may accumulate in a time-dependent manner in the absence of growth, as proposed by Strauss (*26*). Furthermore, because the majority of cells in a tumor may not be in a microenvironment conducive to the rapid growth that occurs in cell culture, the conditions in high-density cultures described here may more closely resemble conditions in the tumor.

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11. Most of the cell lines used in these experiments were obtained from the American Type Culture Collection. MRC-5 was obtained from the Imperial Cancer Research Fund Cell Production Facility. 2774 was the kind gift of T. Kunkel. All cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Mutation rates were determined by the Luria-Delbrück fluctuation test [S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943)]. Inocula containing 100 cells were grown for 2 weeks on 35-mm tissue culture wells. Colonies formed after this time were treated with trypsin and transferred to 100-mm dishes. After 5 days, the replica cultures were plated on 6-thioguanine (5 μg/ml) to determine mutation rate at the *HPRT* locus or on 1 μM ouabain to determine the rate of mutation to resistance to this drug. After 2 weeks, plates were stained and colonies larger than 50 cells were counted. The mutation rate was calculated by the method of the mean [R. L. Capizzi and J. W. Jameson, *Mutat. Res.* **17**, 147 (1973)].
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18. B. Richards, unpublished data. The rate of mutation to Oua^R was elevated 24- to 35-fold in tumor cells deficient in other mismatch repair components.
19. Analysis of *HPRT* mutations was performed as in (*20*), except that an ABI automated sequencer (Model 377) was used to determine the base alterations. Positions of mutations in the coding sequence [D. Jolly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 477 (1983)] are presented with the A in the ATG initiation codon being nucleotide 1. Mutations in the introns that alter splicing are numbered with respect to the *HPRT* genomic sequence [A. Edwards *et al.*, *Genomics* **6**, 593 (1990)].
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21. Microsatellite instability was analyzed as in (*20*). DNA purified from cultures grown from single cells of the indicated lines was used to amplify microsatellite loci. Four loci were assayed: *BAT26*, which contains a mononucleotide run; *D10S197* and *D16S521*, which are both dinucleotide repeats; and *D7S1794*, which contains a trinucleotide repeat. The frequency of instability presented in the text represents the fraction of subclones from each of the lines exhibiting novel alleles at the loci. Patterns of alleles at one of the microsatellite loci (*D10S197*) in single cell clones isolated from 2774 are presented in Fig. 1.
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TECHNICAL COMMENTS

Observations of Emission Bands in Comet Hale-Bopp

Heike Rauer *et al.* observed anomalous H_2O^+ and NH_2 emission bands in comet Hale-Bopp (C/1995 01) at large heliocentric distances before perihelion (*1*). The subject bands are from bending vibrational transitions, $(0,\upsilon_2',0)$-$(0,0,0)$, of the $\tilde{A}^2A_1 - \tilde{X}^2B_1$ system of the two isoelectronic molecular species. Except at the shortest heliocentric distances studied, only emissions from even bending vibrational levels were observed, a phenomenon for which existing fluorescence excitation models provide no explanation, according to Rauer *et al.*

An equivalent observation, however, was made in comet Kohoutek by Wehinger *et al.* (*2*), who identified H_2O^+ and attributed the phenomenon to fluorescence excitation of molecules at temperatures below 50 K. The reason for the missing vibrational bands can be found in the electronic structure of H_2O^+ and NH_2 (*3*, *4*) that gives rise to optical transitions involving a lower state bent asymmetric rotor with quantum numbers J'', N'', K_a'', and K_c'' and an upper state linear symmetric rotor with quantum numbers J', N', and K', the latter quantum number being equivalent to K_a'. While there is no vibrational level-dependent constraint on K_a'' values in the ground electronic state, odd K' vibronic sublevels of the linear excited state are restricted to even bending vibrational states, while even K' sublevels are associated with odd bender states.

Given the $\Delta K_a = \pm 1$ selection rule of the H_2O^+ and NH_2 $\tilde{A}^2A_1 - \tilde{X}^2B_1$ transitions, absorption to the unobserved odd

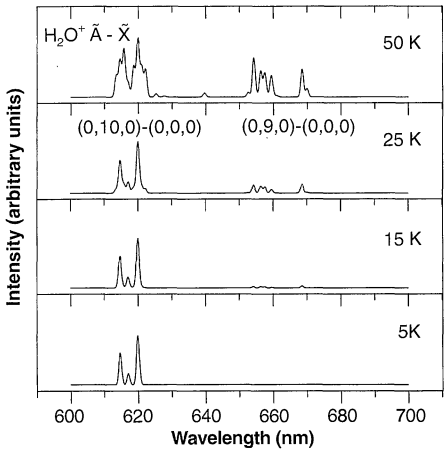

Fig. 1. Fluorescence excitation spectra calculated for the H_2O^+ $\tilde{A}^2A_1 - \tilde{X}^2B_1$ system at four temperatures of the ground-state molecule. Calculation assumes 1-nm resolution and a uniform spectral sensitivity.