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22. These inward rectifying and nonrectifying curves could be reproduced by measuring an individual ganglion cell's response to pressure ejection of ACh (n = 5 cells from two retinas) or ACh plus GABA (n = 2 cells from 1 retina), respectively (D. P. Wellis, M. B. Feller, C. J. Shatz, F. S. Werblin, unpublished data).
23. There is evidence in rat hippocampus that *d*-tubocurarine can act as a weak competitive antagonist for $GABA_A$ receptors [F. J. Lebeda, J. J. Hablitz, D. Johnston, *J. Neurophysiol.* **48**, 622 (1982)], implying that our results could be due to a pharmacological blockade of $GABA_A$ receptors and not neuronal nAChRs. However, it has been shown that nicotine has no cross-reactivity with $GABA_A$ receptors [V. E. Wotring and W. Yoon, *Neuroscience* **67**, 293 (1995)], implying that the blockade of waves we see in the presence of nicotine is due to desensitization of nAChRs. This conclusion, combined with the rectifying characteristics of I-V relationship of the compound PSCs seen in the presence of SR95531 implies that the *d*-tubocurarine is acting directly on nAChRs. In addition, in intermediate lobe cells of the porcine pituitary, although the $GABA_A$ antagonist bicuculline acts as a competitive antagonist at nAChRs, SR95531 does not [Z. W. Zhang and P. Feltz, *Br. J. Pharmacol.* **102**, 19 (1991)]. In view of the specific effect that SR95531 has on the chloride contribution to the periodic compound PSCs, we can conclude SR95531 acts specifically on $GABA_A$ receptors. Although $GABA_A$ activated Cl^- conductances are triggered during a wave, their contribution to the propagation of the wave may depend critically on how much these conductances excite the cell. In several kinds of immature neurons, GABA can have excitatory effects [K. J. Staley, B. L. Soldo, W. R. Proctor, *Science* **269**, 977 (1995); E. Cherubini, J. L. Gaiarsa, Y. Ben-Ari, *Trends Neurosci.* **14** (12), 515, (1991)]; however, the GABA response has not been characterized for retinal ganglion cells.
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30. A recently proposed model [P. Y. Burgi and N. M. Grzywacz, *J. Neurosci.* **14**, 7426 (1994)] was based on the assumption that the spread of excitation was through extracellular potassium. This model is inconsistent with our results because blocking K^+ currents with intracellular Cs^+ had no effect on the periodic compound PSCs. In addition, the reversal potential for the compound PSCs was not consistent with a K^+ conductance.
31. This model is consistent with the observations that amacrine cells in the developing rabbit retina have functional nAChRs [R. O. Wong, *J. Neurosci.* **15**, 2696 (1995)] and that, in the developing chick retina, amacrine cells have the α-bungarotoxin–insensitive form of the neuronal nAChR [D. E. Hamassaki-Britto *et al.*, *J. Comp. Neurol.* **347**, 161 (1994); K. T. Keyser *et al.*, *J. Neurosci.* **13**, 442 (1993)].
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Polyclonal Origin of Colonic Adenomas in an XO/XY Patient with FAP

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It is widely accepted that tumors are monoclonal in origin, arising from a mutation or series of mutations in a single cell and its descendants. The clonal origin of colonic adenomas and uninvolved intestinal mucosa from an XO/XY mosaic individual with familial adenomatous polyposis (FAP) was examined directly by in situ hybridization with Y chromosome probes. In this patient, the crypts of the small and large intestine were clonal, but at least 76 percent of the microadenomas were polyclonal in origin.

Investigations of clonality in the normal intestinal mucosa have centered on the examination of laboratory mice (*1*–*3*). In the neonatal mouse, intestinal crypts are polyclonal, but through a process known as "purification," crypts become monoclonal by the time mice are 2 weeks old (*4*). In the normal human intestine, investigation has been limited by the lack of suitable markers of clonality. Clonality studies are of great relevance to our understanding of the process of tumorigenesis. Proponents of mutational theories of tumor development suggest that tumors arise from a series of mutations occurring in one cell and its progeny (*5*–*7*). Others have argued that tumors are not clonal in origin but require the interaction of multiple cells (*8*, *9*) and that outgrowth of a dominant clone during subsequent development accounts for their apparent monoclonality.

It is possible to differentiate between XY cells and XX or XO cells by in situ hybridization with chromosome-specific probes; thus, the Y chromosome can be used to determine patch size and tissue clonality, as in XX/XY chimeric mice (*10*). To investigate clonality in the human intestine, we studied tissue from an XO/XY mosaic individual of male phenotype who, by coincidence, also had familial adenomatous polyposis (FAP). This was a serendipitous discovery, as the chance of finding such an individual is probably less than one in a hundred million. The clinical diagnosis of FAP was confirmed by mutation analysis of the patient's *APC* gene: A protein truncation test and subsequent sequence analysis (*11*) revealed a germline frameshift mutation at codon 1309. The patient had undergone a prophylactic total colectomy at 32 years of age, from which paraffin-embedded material was available.

We first performed karyotyping and fluorescent in situ hybridization (FISH) (*12*), using the Y chromosome-specific probes cY97 and pDP105 (*13*), on the patient's peripheral blood lymphocytes (PBLs). These tests confirmed that he was a mosaic and also showed the Y chromosome to be dicentric. The karyotype was 45,X/46,X, dic(Y)(Ypter → cen → Yq11.23:: Yp11.3 → cen → Yq11.23). FISH demonstrated that approximately 20% of the PBLs were XO.

Nonisotopic in situ hybridization (NISH) (*14*) was then performed on histological sections of the small and large intestine, with the use of the Y chromosome–

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specific probes (Fig. 1). Intestinal crypts were composed almost exclusively of either XY or XO cells (Fig. 1C). Scattered Y-negative nuclei were seen in otherwise Y-positive crypts. These Y-negative nuclei are artifacts of tissue sectioning, which produces partial nuclei that do not all contain the Y chromosome (*10*) (counts from male control sections showed that approximately one in five nuclei appear Y-negative). Scattered Y-positive nuclei were also seen in otherwise Y-negative crypts; however, combined immunostaining and NISH showed that these positive nuclei belonged to leukocytes infiltrating the crypt epithelium (Fig. 1D). Overall, 9% of the crypts were XO; however, this value ranged from 24% in the terminal ileum to 3.4% in the distal colon. Of more than 1000 crypts examined at patch borders from seven blocks of the terminal ileum and colon, no mixed XO/XY crypts were seen. Villus epithelium showed a mixture of XO and XY cells as expected (*1*) (Fig. 1F).

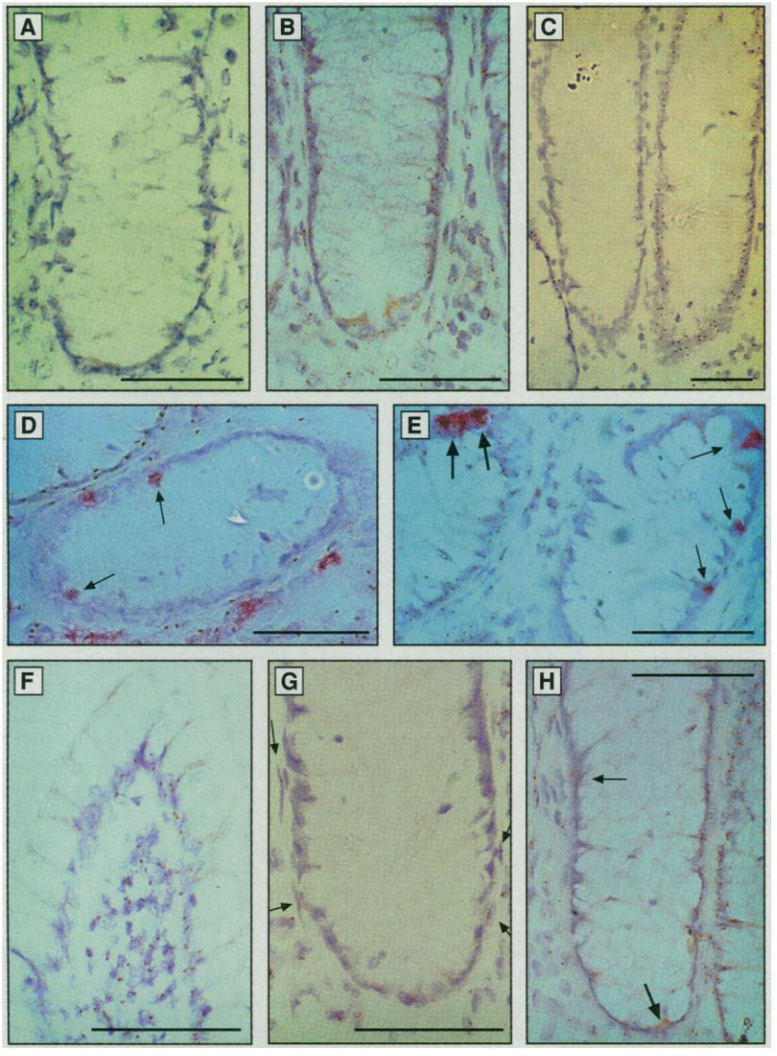

Fig. 1. NISH staining of normal intestinal mucosa (*14*). Nuclei were counterstained with hematoxylin. NISH was performed on 4-μm sections from seven blocks of colon from four control male FAP patients, and from seven blocks of small and large intestine from the XO/XY mosaic individual. (**A**) Colonic section from a control male FAP patient stained with the Y probes cY97 and pDP105; cells show a dark spot over the nucleus. (**B**) Colonic section from the XO/XY mosaic patient stained with a control X centromere probe, pSV2X5 (*27*). (**C**) Colonic section from the XO/XY mosaic patient stained with Y probes, showing an XY crypt and an adjacent XO crypt (no stain). (**D**) Y-positive leukocytes infiltrating the colonic crypt epithelium (arrows) visualized by combined immunostaining for CD45 [with monoclonal mouse anti-human leukocyte common antigen (Dakopatts)] and NISH. (**E**) XY colonic crypt with Y-positive neuroendocrine cells (large arrows) and an adjacent XO crypt with Y-negative neuroendocrine cells (small arrows) visualized by combined immunostaining for chromogranin A [with monoclonal mouse anti-human chromogranin A (Dakopatts)] and NISH. (**F**) A villus from the terminal ileum showing that the epithelium is of mixed XO and XY type; the villus population is derived from the migrating cells of several crypts (*1*). (**G**) Y-positive pericryptal myofibroblasts (arrows) surrounding an XO colonic crypt. (**H**) A mixed XO/XY colonic crypt stained for chromogranin A and NISH; the endocrine cell at the crypt base is positive (large arrow), but in the left hemicrypt the endocrine cell is negative (small arrow). Scale bars, 50 μm.

A combination of immunostaining and NISH was required to show that crypt neuroendocrine cells shared the same genotype as other resident crypt cells (Fig. 1E). In XY crypts, 885 of 1071 (83%) neuroendocrine cells counted were Y-positive, whereas in XO crypts, only 3 of 92 (3%) neuroendocrine cells were Y-positive. These three Y-positive cells were probably false positives, owing to closely applied Y-positive pericryptal cells or leukocytes (*10*). These data strongly suggest that human intestinal crypts are clonal populations, each derived ultimately from a single stem cell; neuroendocrine cells share this derivation, so it is unlikely that they arise from the neuroectoderm, as hypothesized by others (*15*). There was no relation between the staining patterns of the pericryptal sheath cells and the crypt epithelial cells (Fig. 1G), which suggests that these cells segregate independently.

From a total of 12,614 crypts examined, 4 mixed XO/XY crypts were seen in XY patches (Fig. 1H). These crypts may have resulted from failure of crypt purification, although mixed crypts have been reported previously in the aging human colon (*16*) and in mice given mutagens (*17*). In this patient, they were probably due to nondisjunction, with loss of the Y chromosome in one of the crypt's stem cells.

The patient's colon contained thousands of tubular adenomas, ranging in size from monocryptal adenomas to microadenomas 2.5 mm in diameter; no larger adenomatous polyps were seen. If an adenoma were of clonal origin, all dysplastic crypts (*18*) within it would be entirely XO or entirely XY. The NISH staining showed that monocryptal adenomas were entirely XO or XY (Fig. 2A), with no mixed pattern. Excluding monocryptal adenomas, 246 (94%) of the microadenomas contained exclusively XY dysplastic crypts and 4 (2%) contained entirely XO dysplastic crypts. However, 13 (5%) of the microadenomas contained a mixture of XO and XY dysplastic crypts (Fig. 2, B and C). Thus, of the 17 adenomas containing XO dysplastic crypts, 13 (76%) were of mixed XO/XY type and 4 (24%) were purely of XO genotype.

From the relative frequencies of XO/XY and XO-only adenomas, we estimate that a minimum of 76% of adenomas (above monocryptal size) were polyclonal in origin. No individual dysplastic crypts of mixed XO/XY type were seen. About 45% of the XY adenomas contained entrapped XO crypts of normal morphology (Fig. 2D). Examination of 3707 normal colonic crypts from male FAP patients stained with Y

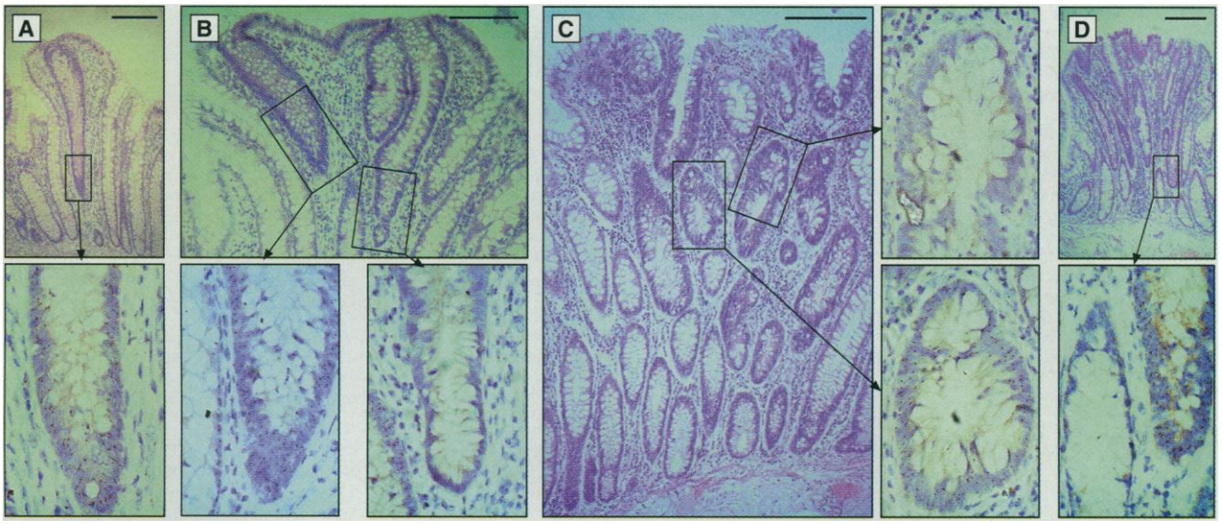

Fig. 2. Adenomatous polyps from the mosaic patient visualized by hematoxylin and eosin and NISH with Y probes. (**A**) An XY monocryptal adenoma. (**B**) A small microadenoma showing an XO dysplastic crypt (right) and an XY dysplastic crypt (left). (**C**) A microadenoma showing an XO dysplastic crypt (top) and an XY dysplastic crypt (bottom). (**D**) An XY microadenoma showing an entrapped normal XO crypt adjacent to an XY dysplastic crypt. Scale bars, 200 μm.

probes revealed only a single XO crypt. Similar examination of 66 adenomatous polyps, ranging in size from bicryptal adenomas to 1-cm adenomas, showed no XO-only adenomas or mixed XO/XY adenomas. Thus, nondisjunction of the Y-chromosome is a rare event in the normal intestinal crypt and in colorectal adenomas.

Our observation that normal human intestinal crypts are monoclonal agrees with earlier mouse work, but our observation that colonic tumors can be polyclonal conflicts with current models of tumorigenesis. The clonality of human tumors has been explored with X-linked markers, but in colorectal tumors this approach has produced conflicting results (*19–21*). Investigations in chimeric mice have shown that small, carcinogen-induced dysplastic foci were monoclonal, but that 2 of 17 larger lesions were polyclonal, these foci being explained as "collision" tumors (*22*).

There are three plausible explanations for our results. (i) Adenomas may be truly polyclonal. "Field effects" may cause adenomas to cluster (nonrandom collision); multiple adenomatous clones may be required for (or strongly favor) early adenoma growth; or, perhaps more likely, early adenomas may induce adenomatous growth in surrounding crypts (this may apply especially in APC patients because all cells already have a single FAP mutation). (ii) The XO/XY adenomas may actually be XY adenomas that have focally lost their Y chromosome. The Y chromosome is frequently lost in a variety of carcinomas (*23*), but in colorectal tumorigenesis this event usually occurs after *P53* mutation in the late stages of adenoma development and in carcinomas (*24*). Also, our examination of adenomas from male FAP control individuals did not reveal a single focus of Y-chromosome loss, even though these adenomas were larger than those in the XO/XY patient. However, it should be noted that the mosaic patient has an abnormal Y chromosome, so such chromosomal loss may occur more readily. (iii) Polyclonality may result from random collisions between adenomas. The large number of polyps in patients with FAP means that some collisions are likely to occur, and the frequency of these collision tumors can be modeled mathematically. The results of statistical analyses, however, show that collision tumors are unlikely to account for the high proportion of polyclonality we observed (*25*). Furthermore, there was no association between adenoma size and the presence of XO/XY polyclonality, as might be expected from random collision of tumors (*26*). Overall, our results support the first hypothesis—that the adenomas in this patient are truly polyclonal—with resulting implications for models of tumorigenesis.

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25. In general, assume that n adenomas are distributed randomly along a one-dimensional colon containing y crypts. Assume that before collision, each polyp has width x. Then the number of interadenoma spacings that are less than x (that is, the number of collisions between polyps) is approximately binomially distribut-

ed with probability, $p = 1 - e^{-nx/y}$, so that the expected number of collisions is np and variance is $np(1 - p)$ [R. Pyke, *J. R. Stat. Soc. B* **27**, 395 (1965)]. Our data suggest that 13 out of 17 (76%) adenomas were polyclonal. We observed approximately 300 polyps in a colon length of 15,000 crypts. The mean width of the observed adenomas was seven crypts (although this figure is an overestimate of x, because it was observed after any collisions had occurred, which presumably had the effect of increasing x). We analyzed the model assuming different values of n and determining whether these values can account for two observations: (i) the final figure of 300 adenomas after any collisions have occurred, and (ii) the estimate of 76% polyclonality. In general, it is not possible to reconcile observations (i) and (ii). If n is sufficiently small to account for observation (i), then far too few collisions occur to account for observation (ii). Conversely, if n is large enough to account for observation (ii), then many more polyps than 300 result. For example, use an estimate for n, assuming that each polyclonal adenoma is formed of three original adenomas. In this case, $n = 756$, $x = 7$, and $y = 15{,}000$. Then, it follows that $p = 0.297$; np (number of collisions) = 225; $np(1 - p)$ (variance in the number of collisions) = 157 (SD = 12.5); and $n(1 - p)$ (number of noncollision polyps) = 531. If each collision involves a mean of three adenomas, then 225/3 = 75 polyclonal adenomas result (12.4% of total) and a total of 606 adenomas is predicted. Even if the number of collisions is increased by 2 SD (~5% confidence limit) from the 225 predicted, the total number of polyps resulting is 590, which is far in excess of 300. Moreover, in order to account for the observation that 13 out of 17 adenomas were polyclonal, given that 12.4% of all polyps were polyclonal, the appropriate terms in the binomial distribution ($p = 0.124$, $n = 17$) are calculated to give P (observed polyclonality) $< 3 \times 10^{-9}$. Thus, the model suggests that the collision hypothesis cannot account for the observed data.

26. For mixed (XO/XY) adenomas, mean width = 5.93 crypts ($n = 13$); and for all adenomas, mean width = 6.82 ($n = 285$) and SEM = 0.554. The width of mixed adenomas does not differ significantly from that of the general polyp population (normal distribution, two-tailed test, $P > 0.1$).
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TECHNICAL COMMENTS

X Chromosome Dosage Compensation in *Drosophila*

In the recent article by Richard L. Kelley and Mitzi I. Kuroda (*1*) and in an earlier paper (*2*), a model of X chromosome dosage compensation in *Drosophila* was attributed to our laboratory that misrepresents our views. For the record, we briefly summarize our ideas.

Dosage compensation not only occurs in males, but also in other X chromosome genotypes such as metafemales (3X;2A), metamales (1X;3A), and triploid intersexes (2X;3A), where A designates the autosomes (*3*). We have sought to explain the five levels of X expression in males, females, metafemales, triploid metamales, and triploid intersexes. We have proposed that the change in gene dosage in these genotypes is canceled by a trans-acting inverse dosage effect that would also be produced by regulatory genes simultaneously varied. This effect is of the appropriate magnitude to cancel the structural gene dosage effect in all genotypes of the X. The inverse dosage effect has been observed in aneuploids of significant length in both maize and *Drosophila* (*4*, *5*) and produces dosage compensation of many structural genes present on the varied chromosomal segments (*5*, *6*).

As the heteromorphic sex chromosome situation evolved in *Drosophila*, these effects would come into play and produce dosage compensation of most X-linked genes and the doubling of the expression of the autosomes in males. In metafemales, the three X chromosomes are compensated and the autosomal expression is reduced (*7*). Indeed, transgenic copies of the ordinarily X-linked *white* locus, when present on the autosomes, are inversely affected in a dosage series of the X involving males (1X;2A), females (2X;2A), and metafemales (3X;2A) (*8*).

We speculate that the products of the *msl* loci sequester a modifier of chromatin, present in both sexes, to the X chromosome. This situation has evolved to alter the action of the inverse effect in males. That a complex of the MSL proteins localizes to the X chromosome has been elegantly demonstrated by Kuroda and her colleagues (*9*). The binding of the complex requires the presence of all four *msl* gene products, and the synthesis of one of them, *msl-2*, is blocked in females by the product of the *Sex lethal* gene (*2*). The sequestered chromatin modifier is postulated to enhance the action of the inverse effectors to ensure complete compensation of X-linked genes. Because the modifier is sequestered away from the autosomes in males, the tendency for increased expression of the autosomes would be diminished, although some cases of higher autosomal male expression persist (*10*). Therefore, when any of the *msl* loci are mutated, there is no sequestration; the X remains basically compensated and the autosomal expression is increased in general (*11*). The consequent change in chromatin might affect the cytological appearance of the chromosomes in the mutants.

With the Sxl^f mutations, the XX individuals are shifted to male sex determination (*12*) and the MSL complex binds to the two X chromosomes (*13*), thus sequestering more modifying protein from the autosomes and resulting in their lowered expression (*14*). Because the acetylated lysine-16 form of histone 4A is enriched on the male X (*15*), a candidate for the sequestered protein is the responsible histone acetyltransferase [or an inhibitor of a histone deacetylase (*1*)], but there may be other possibilities.

Thus, our explanation, based on gene expression data, proposes a single mechanism of dosage compensation for all X chromosome genotypes with modification by the sex determination mechanism and accommodates the localization of the MSL proteins to the X chromosome in males.

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Response: Because of space limitations, we only briefly referred to the trans-acting inverse dosage effects theory for dosage compensation favored by Birchler and his coworkers (*1*). The controversy can be distilled down to whether the primary defect in *msl* mutant males is an inadequate amount of X-encoded gene products or an excess of autosomally encoded products. Our understanding of Birchler's model is