Preventing Neurodegeneration in the *Drosophila* Mutant *bubblegum*

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The *Drosophila melanogaster* recessive mutant *bubblegum* (*bgm*) exhibits adult neurodegeneration, with marked dilation of photoreceptor axons. The *bubblegum* mutant shows elevated levels of very long chain fatty acids (VLCFAs), as seen in the human disease adrenoleukodystrophy (ALD). In ALD, the excess can be lowered by dietary treatment with "Lorenzo's oil," a mixture of unsaturated fatty acids. Feeding the fly mutant one of the components, glyceryl trioleate oil, blocked the accumulation of excess VLCFAs as well as development of the pathology. Mutant flies thus provide a potential model system for studying mechanisms of neurodegenerative disease and screening drugs for treatment.

Drosophila, with its highly evolved nervous system, short generation time, and amenability to genetic and molecular analysis, offers an incisive tool for the analysis of hereditary neurodegenerative diseases (*1*). For example, the fly mutants *swiss cheese* (*2*), *spongecake*, and *eggroll* (*3*) show late-onset neurodegeneration in the adult brain resembling that seen in various human diseases.

In screening for *Drosophila* brain degeneration by P-element insertion mutagenesis (*4*), we isolated *bubblegum*, a mutant gene on chromosome 2. Upon cloning the gene, we found that it was similar to vertebrate VLCFA acyl coenzyme A (CoA) synthetase, suggesting possible relevance of the fly mutant to human ALD, which is associated with excess VLCFAs due to mutations in a transporter gene for that enzyme. We therefore examined the VLCFA profile in the fly mutant and found that it was also abnormal.

The development of neuropathology with age in the visual system of *bubblegum* flies was examined by light and electron microscopy (Fig. 1) (*5*). In the young fly mutant, the optic lobes appear normal but, with age, regionally specific degeneration develops that is not seen in the parental strain. This is particularly marked in the first optic ganglion, the lamina (corresponding to the vertebrate eye ganglion cell layer), in which photoreceptor axons enter to synapse with second-order neurons. The appellation *bubblegum* refers to the bubbly appearance of the lamina, as seen in light microscope sections (Fig. 1B). There is also degeneration of the cell bodies in the retina. Electron micrographs show inflation of various structures, which is most evident in the expansion in the diameter of the photoreceptor axons (Fig. 1, C and D). This pathology is similar in male and female homozygous *bubblegum* flies.

To identify the affected gene, we isolated DNA adjacent to the P-element insertion by plasmid rescue (*6*). The genomic sequence corresponded to that located at position 34F1-2 on chromosome 2, as determined by the Berkeley *Drosophila* genome project (www.fruitfly.org), and a cDNA clone was obtained from Genome Systems (St. Louis, Missouri). Analysis of the mutant located the P-element insertion between the 5′ untranslated region (5′UTR) and the start codon of an open reading frame of 1947 base pairs (bp) that predicts a protein of 649 amino acids. A BLAST search indicated homology with a VLCFA acyl CoA synthetase expressed in rat liver (*7*) and human brain (*8*) (Fig. 2A). The *bubblegum* gene is the only known VLCFA acyl CoA synthetase–like gene in *Drosophila*.

This enzyme activity is reduced in X-linked human ALD (*9*), which is manifested at a young age by neural demyelination and blindness. In a milder form, referred to as adrenomyeloneuropathy (AMN), onset is later, with progressive paraparesis and distal axonopathy (*9*). The β-oxidation of VLCFAs is catalyzed in peroxisomes, in several steps, after activation to thioester derivatives by the synthetase (*10, 11*). Reduced activity of the enzyme can result in accumulation of excess VLCFAs (*12*), as can a defect in any step of the oxidation process (*13*). ALD and AMN patients have mutations not in the gene encoding VLCFA acyl CoA synthetase, but in that for a member of the ABC transporter superfamily (ALDP), which is thought to be needed for the transport or stabilization of the enzyme (*14*). According to the pathway (Fig. 2D), mutations in the synthetase gene, as in *bubblegum*, would be expected to cause the same disturbance as those affecting the transporter.

To study *bubblegum*, we analyzed fatty acids from 15-day-old whole flies by gas chromatography (*15*), comparing homozygous mutant flies with the parent strain. Mutant males indeed showed increased VLCFAs, including C_{22}, C_{24}, and C_{26}, whereas those of chain length below C_{20} did not increase (Fig. 3A). Paradoxically, although both male and female mutant flies

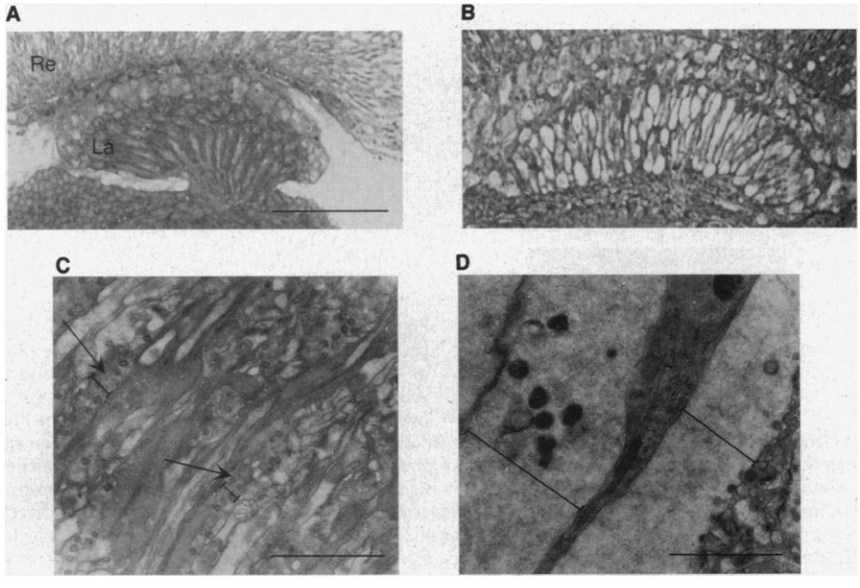

Fig. 1. Optic lobe degeneration in *bubblegum* mutant flies. (A and B) Shown are horizontal sections of plastic-embedded adult male heads, stained with toluidine blue. (**A**) Young mutant fly (1 day old). (**B**) A 15-day-old mutant. Note bubbly appearance of the lamina. La, lamina; Re, retina. Bar, 50 μm. (C and D) Electron micrographs. (**C**) Lamina of 1-day-old *bubblegum* male, sectioned parallel to the photoreceptor axons (arrows). The axon diameter (indicated by brackets) is the same as in normal flies (not shown). (**D**) At 15 days of age, the mutant photoreceptor axons have become greatly dilated. Bar, 2 μm.

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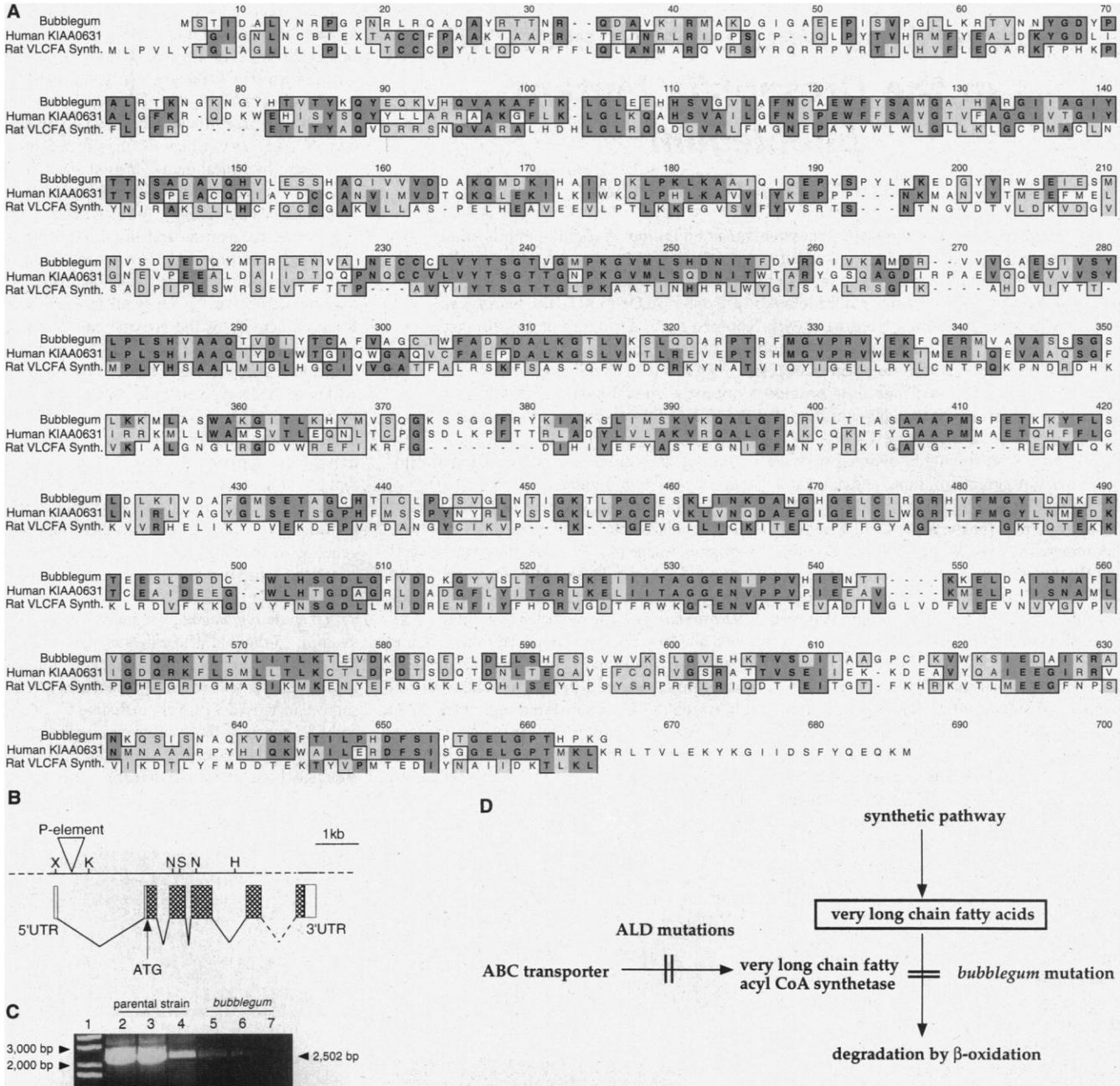

Fig. 2. The *bubblegum* gene and metabolic pathway for VLCFAs. (**A**) Alignment of the *bubblegum* protein with rat VLCFA acyl CoA synthetase (Rat VLCFA Synth.) and a human brain protein sequence of as yet unidentified function. Identical and similar amino acids are identified by dark and light shading, respectively. Compared with the rat synthetase, *bubblegum* has 15% identity and 32% overall similarity, but the homology with the human sequence is greater: 40% and 61%, respectively. (**B**) Genomic map of the *bubblegum* gene region. Triangle indicates the P-element insertion. Open and hatched rectangles indicate untranslated and coding sequences, respectively. The exon-intron structure of the 3′ region has not been determined. Restriction enzyme site: H, Hind III; K,Kpn I; N, Nco I; S, Sal I; X, Xho I. (**C**) Transcription of the *bubblegum* gene is greatly reduced in the mutant fly. Reverse transcription–polymerase chain reaction, using 1 μg of total RNA from the parental strain (lanes 2 through 4) or *bubblegum* (lanes 5 through7), was used to detect the *bubblegum* transcript. The two primers used were chosen from the 5′UTR and 3′UTR sequences. Lanes 2 and 5, whole fly; lanes 3 and 6, bodies only; lanes 4 and 7, heads only. (**D**) Normally, the synthetase activates VLCFAs for degradation. In ALD patients, a genetic defect in an ABC transporter interferes with the function of the synthetase. As a consequence, there is an excess accumulation of VLCFAs. In the *bubblegum* mutant, a similar effect is due to a mutation in the synthetase itself.

showed similar neuropathology, homozygous females showed little change in VLCFA levels, presumably because of differences in the metabolism of VLCFAs (Fig. 3B).

In human ALD, dietary treatment has been used to restore VLCFAs to normal levels. Feeding patients a monounsaturated fatty acid, glyceryl trioleate oil (GTO), reduced the level of saturated C_{26} in plasma by about 50% within 4 months (*16*). So-called "Lorenzo's oil," a combination of GTO with glyceryl trierucate oil, normalized C_{26} in a month (*17*). These monounsaturated fatty acids probably compete in the fatty acid elongation system, reducing the for-

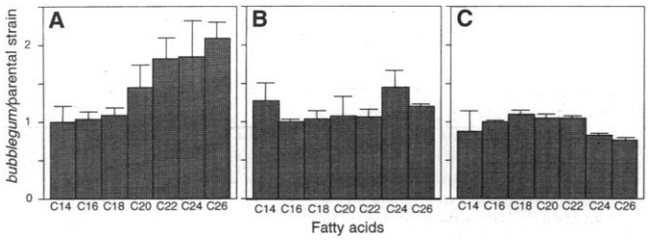

Fig. 3. Preventing the accumulation of VLCFAs in *bubblegum* males. Profile of adult *bubblegum* (**A**) males and (**B**) females raised for 15 days in ordinary medium. (**C**) Adult males that were raised in medium containing 2.5% GTO from the first larval instar through to 15 days of adult age. Each bar represents the ratio of the homozygous *bubblegum* mutant to the parental strain, based on four experiments, each using 100 flies. Standard deviations are shown.

mation of the saturated VLCFAs (*18*). We therefore tested whether similar dietary treatment can reduce the concentration of C_{26} and prevent the pathology seen in *bubblegum*.

When larvae were raised on the usual cornmeal-yeast-agar medium and the emerging adults transferred to medium supplemented with 2.5% GTO, the lamina was still somewhat abnormal (Fig. 4A). Considering that oil treatment in ALD could not prevent progression of the disease once patients had shown neurological symptoms (*17*), we tried beginning treatment with the oil at preadult stages, continuing into adulthood. The amounts of VLCFAs in male flies were reduced to normal (Fig. 3C). In addition, degeneration was prevented (Fig. 4B), both in male and female mutant flies. At the temperature used (29°C to accelerate aging), the average life-span was 19 days for the parental strain and 13 days for *bubblegum*; GTO treatment restored the life-span of *bubblegum* to that of normal flies. The oil had no evident deleterious effect on normal flies.

In ALD, visual loss occurs (*9*), and anatomical analysis has shown abnormalities of the optic nerve and degeneration of the ganglion cell layer (*19*). We therefore tested the vision of *bubblegum*. Flies transformed with P-elements marked with the mini-*white*$^+$ gene, as in *bubblegum*, express some eye pigment and are phototactic (*20*). We used the countercurrent phototaxis test, in which a population of flies is fractionated according to number of positive responses in repeated trials in movement toward light (*21*). As seen in Fig. 4C, the mutants raised in ordinary medium showed poor response (flies responding in 10% of all trials), consistent with their visual system abnormalities, whereas those raised with GTO from the larval stage onward showed greatly improved phototaxis (responding in 60% of all trials).

The difference between male and female mutant flies homozygous for the second chromosome P-element insertion is curious. Females showed the same age-dependent degeneration, which was prevented by feeding the oil, despite not showing the excess VLCFAs seen in males. The mutation in the synthetase is sufficient to produce the pathology, but not to cause a high level of VLCFAs in both sexes, implying that other, sex-related factors influence the accumulation of VLCFAs.

In X-linked human ALD (*9*) and in three knockout mice of the ABC transporter gene (*22–24*), there was no correlation between the amount of VLCFAs and the severity of pathology. Some individuals with high VLCFAs escaped the neurological defects (*9*), and the knockout mice did not show the pathology of ALD, in spite of having elevated VLCFAs. These observations suggest that excess VLCFAs and the pathology may not have a direct causal relationship, but may be separate ramifications of another, underlying defect. H. W. Moser *et al.* (*9*) suggested that autosomal modifer genes may play a role in the pathogenesis of ALD. The differences in our observations between males and females are consistent with that interpretation. In *Drosophila*, it should be possible to do genetic analysis to clarify this problem by identification of suppressor and enhancer genes.

The majority of human ALD mutations have been traced to the transporter gene. The fact that mutations in the synthetase gene in *bubblegum* produce effects that have some features in common with ALD suggests that additional screening of patients and their families for mutations in the human synthetase gene might be desirable. Knockout of the transporter gene in mice has so far not resulted in an ALD phenotype (*22–24*). It remains to be seen whether knockout of the synthetase or double knockout of both the enzyme and the transporter will do so.

The pathological phenotype in *bubblegum* is prevented by feeding the oil. Although ALD patients treated with "Lorenzo's oil" have experienced a reduction in the amount of C_{26}, there has been little success in preventing progression of the disease, perhaps due to the late start of treatment, which also failed to rescue *bubblegum*. Because experiments on optimization of treatment are difficult to perform on human subjects, it is possible that fly mutants such as *bubblegum* can serve as useful model systems for rapidly screening food additives, drugs, and regimens of administration.

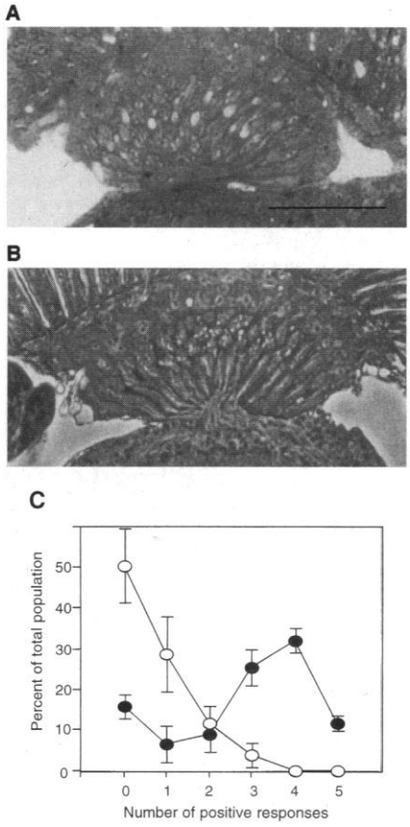

Fig. 4. Preventing degeneration in *bubblegum* by early initiation of feeding with GTO. Sections and staining are as in Fig. 1. (**A**) Late initiation. Homozygous male *bubblegum* that were placed in GTO-containing medium for 15 days following their emergence as adults still showed considerable degeneration in the lamina. (**B**) For homozygous male *bubblegum* that were raised in GTO-containing medium from the larval stage onward through to 15 days of adult age, the late-onset adult degeneration was largely prevented. Bar, 50 μm. (**C**) Rescue of phototactic behavior of *bubblegum* by feeding GTO. In the multiple-trial countercurrent distribution experiment, the ordinate represents flies that responded positively the number of times indicated by the abscissa, out of five trials. Flies raised without GTO (open circles) showed poor performance. Flies pretreated with GTO from the larval stage (solid circles) showed greatly enhanced responses. n = 100 *bubblegum* adult males, 5 days of age, for each graph.

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4. *Drosophila* lines carrying P{*lacZ*, *w*$^+$} were screened for mutations causing reduced life-span at 29°C. Candidates showing reduced life-span compared with the parent strain were examined after aging (but before death) to identify those with brain degeneration (*3*). The *bubblegum* mutant showed such degeneration and was selected for further characterization.
5. Fly heads were prepared by fixation in 1% paraformaldehyde plus 1% glutaraldehyde, postfixation in 1% osmium tetroxide, dehydration in an ethanol series, and embedding in Epon 812. For light microscopy, 1-μm sections were stained with 1% toluidine blue plus 1% Borax. For electron microscopy, ultra-

thin sections (80 nm) were examined with a Philips 201 electron microscope at 60 kV.
6. The presence of a single insertion of the P-element was confirmed by genomic Southern (DNA) blots. Precise excision of the P-element from the mutant restored the normal phenotype.
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Prehistoric Polymers: Rubber Processing in Ancient Mesoamerica

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Ancient Mesoamerican peoples harvested latex from *Castilla elastica*, processed it using liquid extracted from *Ipomoea alba* (a species of morning glory vine), and fashioned rubber balls, hollow rubber figurines, and other rubber artifacts from the resulting material. Chemical and mechanical analyses of the latex and of the processed rubber indicate that the enhanced elastic behavior of the rubber relative to the unprocessed latex is due to purification of the polymer component and to an increase in the strength and number of interchain interactions that are induced by organic compounds present in *I. alba*. These ancient peoples' control over the properties of latex and processed rubber gave rise to the Mesoamerican ball game, a central ritual element in all ancient Mesoamerican societies.

Ancient Mesoamerican peoples were processing rubber by 1600 B.C. (*1*), which predated development of the vulcanization process by 3500 years. They made solid rubber balls, solid and hollow rubber human figurines, wide rubber bands to haft stone ax heads to wooden handles, and other items (*2*). They used liquid rubber for medicines, painted with it, and spattered it on paper that was then burned in ritual. The ball game, played on a ball court with a solid rubber ball, was a key event in ancient Mesoamerican societies (*3*). The *Popol Vuh*, the Maya origin story, captures the game's religious and sacred function by pitting the ball playing skills of the Hero Twins against those of evil lords of the underworld, using complex imagery of human sacrifice, fertility, and regeneration (*4*). Sixteenth-century Spanish invaders reported that apart from its religious significance the ball game also was a sporting event in which contenders gambled for land, slaves, and other valuables.

The raw material for most Mesoamerican rubber balls and for other Mesoamerican rubber artifacts is a latex acquired from the *Castilla elastica* tree (*5*). The tree is indigenous to tropical lowland Mexico and Central America. *Castilla* latex is a sticky white liquid that when dried is too brittle to retain its shape. Sixteenth-century Spaniards relate that ancient Mesoamerican peoples processed the raw material by mixing *C. elastica* latex with juice from *Ipomoea alba* (*6*) (a species of morning glory vine), one chronicler noting that "*ulli* is the resin from a tree that grows in the hot lands . . . when they mix it with another, the resin coagulates" (*7*). Pedro Martyr, the Spanish royal chronicler, commented that "they make these balls from the juice of a certain vine . . . once transformed into a mass they give it the form they desire" (*8*). In the present study, we investigated this processing technology, the extent to which it improves the mechanical properties of latex for balls, rubber bands, and hollow figurines, and the chemical changes responsible for property development.

Rubber artifacts are poorly preserved, but archaeologists have recovered a few hundred ancient Mesoamerican examples. The oldest archaeological specimens are 12 solid rubber balls recovered at the Manatí site in Veracruz, Mexico. The six we examined range from 13 to 30 cm in diameter and weigh from 0.5 to 7 kg. The two oldest Manatí balls date to 1600 B.C., on the basis of radiocarbon dates (*1*). Manatí (1600 to 1200 B.C.) is an Olmec ritual burial site located in a swampy region along the lower Coatzacoalcos River. Other rubber artifacts were dredged from the Cenote Sagrado de Sacrificios, a water-filled limestone sinkhole at the site of Chichén Itzá on the Yucatán Peninsula. Cenote rubber artifacts, dated to between A.D. 850 and 1550 on the basis of stylistic attributes of associated artifacts, include small nodules, small solid balls, wooden tool handles wrapped in rubber, human figurines, human hands, a hollow human head, and a stone tool hafted with a rubber band (*9*).

The earliest dated ball court is the 1400 B.C. (uncalibrated) earthen court at Paso de la Amada, located in the Soconusco region on the coastal plain of Chiapas (*10*). Enclosed masonry ball courts proliferated in the Middle Classic Period (A.D. 400 to 700). By the time of the Spanish invasion of the Americas, versions of a ball game were played in the region from Arizona to northern South America. Several types existed, including one in which players hit a small rubber ball with a bat or stick. The more common hip-ball version of the game was still played in northwestern Mesoamerica until around 1970 (*11*).

We analyzed the Manatí rubber balls (*12*), and in Escuintla, Chiapas, we investigated traditional rubber processing methods. In Escuintla, the huleros (rubber-workers) incised the bark of the *C. elastica* trees and collected the sticky, raw latex in cups placed at the base of the trees. They cut a 5-m length of *I. alba* vine, stripped the leaves and flowers, and wrapped the vine into a coil. After beating and crushing the coiled vine on a rock, they squeezed the juice into a bucket containing latex; about 50 ml of the *I. alba* liquid extract was mixed with about 750 ml of the latex.

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