California has been cooling (*15*). Curiously, their isotope record also points to cooling by ~7°C, although they caution that the actual temperature effect is close to 3°, the rest being an effect related to variations in the history of air masses.

If valid, the contrasting trends in climate between the midwestern and western United States can be reconciled by examining present-day weather systems and how climate is influenced by the position of the jet stream. Whenever the midwestern regions lie on a high-pressure ridge, drawing in warm moisture from the Gulf of Mexico and blocking off the dry, cool continental air masses, the western regions experience milder temperatures, and vice versa. Therefore, if the mid-Holocene period was marked by sustained intrusion of the Gulf of Mexico air mass into the midwestern region, contrasting climatic conditions within the continental United States are indeed conceivable.

REFERENCES AND NOTES

1. Stable isotope compositions are expressed in the δ notation in units of per mil

$$\delta = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000$$

where R = D/H and the standard is standard mean ocean water (SMOW). The δ values of organic materials could be determined with an overall analytical precision better than 2 per mil.
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5. The freeze-dried kerogen was combusted in sealed glass (Vycor) tubes containing cupric oxide at 900°C for 3 hours. Water produced by combustion was cryogenically separated from CO_2 and passed over hot uranium metal to produce hydrogen gas [I. Friedman, *Geochim. Cosmochim. Acta* **4**, 89 (1953)]. Dating of the samples was carried out using the $^{210}Pb_{excess}$ method for the top ~50 cm [S. Krishnaswami *et al.*, *Earth. Planet. Sci. Lett.* **11**, 407 (1971); S. L. Harden, D. J. DeMaster, C. A. Nittrouer, *Mar. Geol.* **193**, 69 (1992)]. Additionally, three ^{14}C dates were obtained on the organic fraction. The ^{14}C dates indicated a linear sedimentation rate of 0.035 cm year^{-1} (r = 0.98) and, when extrapolated to the surface, gave a surface age of 160 years, showing no or negligible contribution from "dead" carbon. Individual samples have been fitted to ages based on the above sedimentation rates based on ^{14}C dates.
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14. As shown in Fig. 4, the weighted mean annual δD (−57 per mil based on a year's sampling) in Kalamazoo precipitation is made up of 7 months of higher (spring and summer) and 5 months of lower (winter) values of δD. It is clear that the annual average can become higher if instead of 7, precipitation of 8 or more months had higher δD values, that is, if the spring and summer seasons were longer than they are today. Similarly, the average δD of precipitation would be lower than today's if the summer and spring seasons were shorter. Changes in the lengths of seasons would be linked to the position of the jet stream and the extent to which the air masses from the Gulf of Mexico reach and sustain in the midwestern parts of the United States. The change in the lengths of seasons will also involve attendant changes in the mean annual temperatures. Thus, periods of higher values of δD will result from an increase in mean annual temperatures and longer spring and summer seasons of increased moisture transport from the Gulf. With several years of analysis, it would be possible to estimate quantitatively the "air mass effect" on the mean annual isotope ratio.
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Requirement for Src Family Protein Tyrosine Kinases in G_2 for Fibroblast Cell Division

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The protein tyrosine kinase c-Src is transiently activated at the transition from the G_2 phase to mitosis in the cell cycle of mammalian fibroblasts. Fyn and Yes, the other members of the Src family present in fibroblasts, were also found to be activated at mitosis. In cells microinjected with a neutralizing antibody specific for Src, Fyn, and Yes (anti-cst.1) during G_2, cell division was inhibited by 75 percent. The block occurred before nuclear envelope breakdown. Antibodies specific for phosphatidylinositol-3 kinase α and phospholipase C-γ1 had no effect. Microinjection of the Src homology 2 (SH2) domain of Fyn was also inhibitory. Functional redundancy between members of the Src family was observed; a Src-specific antibody had no effect in NIH 3T3 cells but inhibited cell division in fibroblasts in which the only functional Src family kinase was Src itself. Thus, Src family kinases and proteins associating with their SH2 domains are required for entry into mitosis.

The proto-oncogene product cSrc and related enzymes are activated during the transition from the G_0 to the G_1 phase of the cell cycle in response to some growth factors (*1–3*), and microinjection studies have shown that they are necessary for some growth factors to transmit mitogenic signals (*4, 5*). c-Src is also activated as fibroblasts enter mitosis (*6*). Mitotic activation of c-Src involves NH_2-terminal serine and threonine phosphorylations—probably by the cyclin-dependent protein kinase Cdc2—concomitant with a net dephosphorylation of Tyr^{527}, the regulatory site (*7*). A mitotic substrate of Src, known as p68 or Sam68, associates with the Src homology 3 (SH3) and SH2 domains of Src (*8–10*). Sam68 is a heterogenous ribonuclear protein (*11*) and may have a function in RNA transport, stability, or splicing. The fact that mitotic substrates of Src can be detected suggests that signal transduction pathways involving tyrosine kinases may function in mitosis. Here, we address whether the Src kinases are required for cell division.

NIH 3T3 cells express three members of the Src family: Src, Fyn, and Yes. To determine whether Fyn and Yes, like Src, become activated during mitosis, we measured their activities in NIH 3T3 cells overexpressing these enzymes, both during interphase and in cells blocked in mitosis by nocodazole treatment (Fig. 1). After immunoprecipitation of Fyn or Yes with antibodies specific to each protein, both autophosphorylation and phosphorylation of enolase were assessed. We observed activation of these enzymes that was three to five times

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their activity in asynchronous extracts; this increase is similar to that observed for Src under the same conditions (*11*). The amounts of expression of Fyn and Yes were unchanged during mitosis (*11*).

The activation of all three members of the Src family at the G_2-mitosis (G_2-M) transition point suggests that they may have a functional role at this stage of the cell cycle. To address this possibility, we microinjected anti-cst.1, a neutralizing antibody specific for Src, Fyn, and Yes (*5*). Quiescent fibroblasts seeded sparsely on cover slips were synchronously stimulated to enter the cell cycle with fetal calf serum (FCS). Analysis with a fluorescence-activated cell sorter showed that after 16 to 18 hours, 70% of the cell population had a 2*n* DNA content and were in G_2 (*12*). The cells were then microinjected with either nonimmune immunoglobulin G (IgG) or anti-cst.1. After incubation for 10 to 12 hours at 37°C, cells were stained for the presence of rabbit IgG and examined. Most cells microinjected with nonimmune IgG had divided, and as a result, pairs of cells containing the rabbit IgG were detected (Fig. 2A). However, most cells microinjected with anti-cst.1 were found sparsely and singly on the cover slip, which indicated that cell division had not taken place (Fig. 2B).

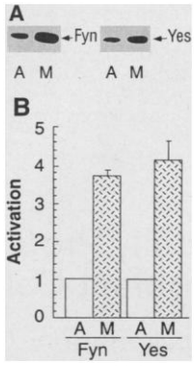

Fig. 1. Activation of Fyn and Yes in mitotically arrested cells. Fyn and c-Yes were immunoprecipitated from interphase (A, asynchronous) or nocodazole-arrested (M, mitotic) cells, and kinase activity was measured (*20*). (**A**) Autophosphorylation. Arrowheads indicate the autophosphorylated kinase. (**B**) Enolase phosphorylation. Activation is relative to the activity in asynchronous extracts, which was given a value of 1. The data were averaged from two separate experiments; values shown are mean ± SD.

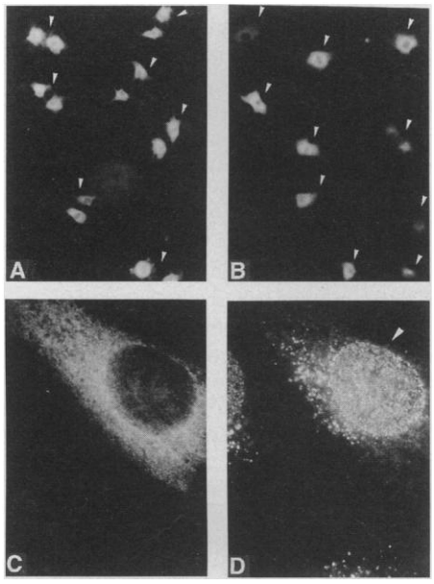

Fig. 2. Arrest of cells microinjected with anti-cst.1 before the onset of mitosis. (**A**) Cells were microinjected with nonimmune IgG fraction during G_2, and after 10 to 12 hours they were stained for antibody-positive cells. (**B**) Cells were microinjected with anti-cst.1 during G_2 and were assessed as in (A). (**C**) A cell microinjected with anti-cst.1 was stained to reveal the microinjected antibody. (**D**) The same cover slip shown in (C) was stained with a mAb to nuclear pore proteins (*21*). In all panels, the white arrowheads mark the positions of injected cells. Original magnifications, ×10 (A and B) and ×63 (C and D).

To test whether the observed effect of the antibody was statistically significant, we microinjected a defined number of cells (usually 50 to 100 in each experiment). After 12 hours, cells containing antibody were counted, and a division index (the ratio of antibody-positive cells to antibody-injected cells) was scored. The division index in control-injected cells was 1.8, but in anti-cst.1–injected cells it was only 1.2, which demonstrated that cell division was inhibited by approximately 75% (Fig. 3). This effect appeared to be specific for the cst.1 epitope because it was at least partially relieved by incubation of the antibody with cognate peptide before microinjection. We also tested antibodies specific for two other signaling molecules, phosphatidylinositol-3 kinase α (PI-3Kα) and phospholipase C–γ1 (PLC-γ1), which are known to be required for the transition from G_1 to S (the period of DNA replication) in response to some growth factors (*13*) and serum (in the case of PLC-γ1) (*12*, *14*); neither antibody inhibited cell division.

To determine the stage of the cell cycle at which the anti-cst.1–injected cells were arrested, we stained them with a monoclonal antibody (mAb) specific for a nuclear pore protein (*15*) (Fig. 2, C and D). Nuclear pores were still detected in the microinjected cells, which demonstrated that the block occurred before the breakdown of the nuclear envelope. In other experiments, we determined that chromatin was not condensed in the anti-cst.1–microinjected cells (*12*), which confirmed that the block occurred before prophase.

Anti-cst.1 recognizes Src, Fyn, and Yes in NIH 3T3 cells (*2*). When we microinjected these cells with an antibody that recognizes only Src (anti-src.1) (*3*), we observed no inhibition of cell division (Fig. 3A). To test whether this was the result of functional redundancy among the Src family kinases, we used "Src fibroblasts" derived from mutant mice that have no Fyn and only a catalytically inactive fragment of Yes (*16*). In cells microinjected with anti-cst.1 the division index was reduced from 1.6 to 1.1; hence, the inhibition was similar to that observed in NIH 3T3 cells. However, although anti-src.1 had no effect in NIH 3T3 cells, it did inhibit cell division in the fibroblasts that lacked catalytically active Fyn and Yes (Fig. 3B). These data demonstrate that Src family kinases share functional redundancy and that at least one member of the Src family is required for cells to exit G_2.

We next investigated whether the SH2 or SH3 domains of the Src family kinases were needed for transition into mitosis. This question could not be addressed by microinjecting cells with plasmids encoding mutant Src molecules because the percentage of microinjected cells that expressed the protein of interest was too small for statistical analysis. We therefore microinjected cells with glutathione-S-transferase (GST) fusion proteins (Fig. 4A). The SH2 domain of Fyn, linked to GST, inhibited cell division by approximately 70%. SH2 domains from guanosine triphosphatase activating protein for Ras (RasGAP), PI-3K, and PLC-γ1 had no effect. We also tested a

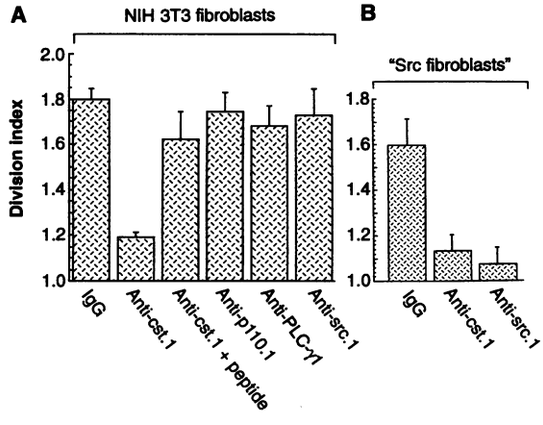

Fig. 3. Requirement for Src family tyrosine kinases for cell division. (**A**) NIH 3T3 cells were microinjected in G_2 with the antibodies shown, and after 10 to 12 hours the number of antibody-positive cells was counted (*22*). (**B**) Fibroblasts lacking active Fyn and Yes ("Src fibroblasts") were microinjected in G_2 with the antibodies shown and were assessed as in (A). For each experiment 50 to 100 cells were microinjected and the division index was calculated. The results from several experiments (n > 5) corresponding to 400 to 1000 injected cells were averaged; values shown are mean ± SD.

series of GST-SH3 constructs and found that none were inhibitory (Fig. 4A) (*12*). To determine when in the cell cycle the block occurred, we stained GST-FynSH2–injected cells with antibodies specific for nuclear lamins (Fig. 4, B and C). This experiment, like the nuclear pore staining (Fig. 2), demonstrated that the nuclear structure was still intact. We conclude that the SH2 domain of Src family kinases is required for their effect before mitosis.

Our data demonstrate that Src family kinases are required for the G_2-M transition. Inactivation of a temperature-sensitive mutant of v-Src during G_2 was found to inhibit mitosis (*17*); however, those cells still contained endogenous, active Src, Fyn, and Yes, so it is not clear that the mechanism of inhibition was the same as we observed here, unless the catalytically inactive protein acted as a dominant negative. Phosphorylation of a tyrosine residue of Cdc2 inhibits the activity of Cdc2 (*18*). Our data suggest that signal transduction cascades involving tyrosine kinases can also have a positive influence on the onset of mitosis. During G_1 many tyrosine kinases, both receptor and nonreceptor types, activate the Ras mitogen-activated protein kinase (MAPK) pathway. In this regard, both a Ras-like protein and a MAPK-like enzyme that appear to function at the G_2-M boundary have been described (*19*), and they both may participate in signaling pathways activated by Src family kinases. Alternatively, because activated Src is known to effect profound changes in the cytoskeleton, it is possible that Src family kinases are required for the cell rounding that precedes mitosis.

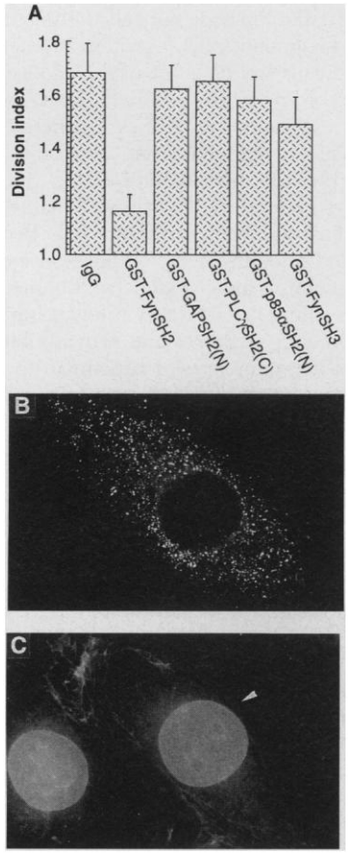

Fig. 4. Inhibition of cell division in cells overexpressing the SH2 domain of Fyn. (**A**) NIH 3T3 cells were microinjected in G_2 with the purified proteins indicated, premixed with control rabbit IgG. After 12 hours, the number of antibody-positive cells was assessed. The results from several experiments ($n > 5$) corresponding to 400 to 1000 injected cells were averaged; values shown are mean ± SD. GST fusion proteins were purified as described in (*23*). Proteins were concentrated up to 3 mg/ml and mixed with nonimmune IgG (final concentration 1 mg/ml) before microinjection. Injected cells were detected as described (Fig. 2). (**B**) A cell microinjected with GST-FynSH2 premixed with fluorescein-conjugated dextran (original magnification, ×63). (**C**) The same cover slip shown in (B) was stained with an antibody specific for nuclear lamins A, B, and C. The arrowhead indicates the microinjected cell.

The Src family kinases apparently phosphorylate proteins whose action is required for the onset of mitosis. One mitotic substrate of Src is Sam68 (*8*, *9*). However, Sam68 is unlikely to be the only critical substrate; it is predominantly a nuclear protein (*11*) and is therefore unlikely to associate with and become phosphorylated by Src family kinases before nuclear envelope breakdown. Also, Sam68 can associate with the SH3 domains of Src and Fyn (*8*, *9*), yet high expression of the SH3 domain of Fyn—which might have been expected to inhibit association of Sam68 with active Src kinases—did not inhibit cell division. Because the SH2 domain of Fyn inhibited cell division, it appears that Src family kinases may be recruited to protein complexes through interactions with tyrosine-phosphorylated proteins. Such complexes could contain critical substrates.

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20. Extracts from NIH 3T3 cells overexpressing (at 5 to 10 times the rate in nontransfected NIH 3T3 cells) human Fyn (*2*) or chicken c-Yes (*11*) were prepared from asynchronously growing cells and from cells blocked in mitosis with nocodazole (40 ng/ml), as described (*9*). Proteins were immunoprecipitated from cell extracts (50 μg) with antibodies to Fyn (fyn.2) and Yes (yes.6), and kinase activity was assessed in vitro by measuring autophosphorylation and enolase phosphorylation (*2*). Anti-yes.5 was raised against amino acids 2 to 17 of the chicken c-Yes sequence and generated as described [R. M. Kypta, A. Hemming, S. A. Courtneidge, *EMBO J.* **7**, 3837 (1988)].
21. NIH 3T3 cells were seeded onto cover slips and grown to 10% confluency, synchronized in G_0 by replacing in serum-free Dulbecco's modified essential medium (DMEM) containing insulin (5 μg/ml), transferrin (5 μg/ml), and antibiotics for 24 to 30 hours, and then stimulated with FCS (15%). After 18 hours, nonimmune IgG fraction or anti-cst.1 was microinjected into the cytoplasm. Cells were incubated for 10 hours, washed once with phosphate-buffered saline, and fixed for 5 min with ice-cold methanol. Cells containing antibody were detected by incubation with fluorescein-conjugated goat antibody to rabbit IgG diluted 1:100 in phosphate-buffered saline for 30 min. Nuclear pore staining was done with mAb 414, a mAb to a family of nuclear pore proteins (*15*), diluted 1:4000 in phosphate-buffered saline containing FCS (5%) for 30 min; cells were washed three times in phosphate-buffered saline and incubated with Texas Red–conjugated goat antibody to mouse IgG. All cover slips were finally washed in phosphate-buffered saline containing Hoechst 33258 (Sigma, final concentration 1 μg/ml), rinsed in water, inverted, mounted in Moviol (Hoechst) on glass slides, and viewed with an Axiophot fluorescent microscope. Anti-cst.1 was raised against peptide Tyr-Gln-Pro-Gly-Glu-Asn-Leu, which occurs in the COOH-termini of Src, Fyn, and Yes (*2*), and affinity-purified as described (*5*). Nonimmune IgG was purified from normal rabbit serum on a protein G column (Pharmacia). Antibodies were concentrated up to 3 mg/ml in a Minicon concentrator (Amicon).
22. Cells were seeded on cover slips, synchronized in G_2, and microinjected with affinity-purified antibody or antibody that had been incubated with its cognate peptide; after 10 to 12 hours, antibody-positive cells were visualized as described (Fig. 2). Anti-p110.1 recognizes the last 15 amino acids of the p110 catalytic subunit of bovine brain PI-3Kα (*13*); anti-src.1 recognizes amino acids 40 to 58 in the unique domain of the mouse c-Src sequence (*3*); and anti–PLC-γ1 was raised against the GST fusion protein bearing both SH2 domains of human PLC-γ1. Antibodies were affinity-purified and concentrated up to 3 mg/ml as described (*5*).
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