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14. ASK1 cDNA was introduced into a yeast expression plasmid pNV11 [H. Shibuya *et al.*, *Nature* **357**, 700 (1992)]. SHO1 is an SH3 domain-containing transmembrane osmosensor that constitutes another signaling pathway leading to hyperosmolarity responses by way of HOG1 activation independently of SSK2 or SSK22 (13). Single or double mutant strains of SHO1, SSK2, or SSK22 are resistant to hyperosmotic medium; however, strains with defects in SHO1, SSK2, and SSK22 are unable to grow in hyperosmotic medium.
15. ASK1 could not restore the osmotic response in a PBS2 [downstream target of SHO1, SSK2, and SSK22 (13)]-defective yeast strain (K. Irie and K. Matsumoto, unpublished data), which indicates that ASK1 activity observed in TM257-H1 was mediated by the PBS2-HOG1 signaling pathway.
16. J. Rouse *et al.*, *Cell* **78**, 1027 (1994).
17. J. Han, J.-D. Lee, L. Bibbs, R. J. Ulevitch, *Science* **265**, 808 (1994).
18. J. C. Lee *et al.*, *Nature* **372**, 739 (1994).
19. *Xenopus* MAPK [Y. Gotoh *et al.*, *EMBO J.* **10**, 2661 (1991)] and *Xenopus* MAPKK (34) were cloned as described. Coding regions for rat SAPK $\alpha$  (4), human p38 [J. Han, B. Richter, Z. Li, V. V. Kravchenko, R. J. Ulevitch, *Biochim. Biophys. Acta* **1265**, 224 (1995)], mouse SEK1 (5), and human MKK3 (6) were amplified by PCR. An HA tag was introduced into the Bgl II and Eco RI sites of a mammalian expression vector pSR $\alpha$ 456 [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)], yielding pSR $\alpha$ -HA1. The cDNAs encoding MAPK, SAPK $\alpha$ , p38, MAPKK, SEK1, and MKK3 were subcloned into the Bgl II site of pSR $\alpha$ -HA1. ASK1 cDNA was introduced into another mammalian expression vector, pcDNA3 (Invitrogen). For transient expression, COS7 cells were transfected with lipofectamine (Life Technologies) according to the manufacturer's instructions. For preparing extracts, cells were lysed in a buffer solution containing 20 mM tris-HCl (pH 7.5), 12 mM  $\beta$ -glycerophosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM dithiothreitol (DTT), 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (20  $\mu$ g/ml). Cell extracts were clarified by centrifugation at 15,000g for 10 min. For immunoprecipitation, the supernatants were incubated with polyclonal anti-serum to ASK1 (24) or monoclonal antibody to HA (12CA5) for 1 hour at 4°C. After the addition of protein A-Sepharose (Pharmacia Biotech), the lysates were incubated for an additional 1 hour. The beads were washed twice with a solution containing 500 mM NaCl, 20 mM tris-HCl (pH 7.5), 5 mM EGTA, 1% Triton X-100, 2 mM DTT, and 1 mM PMSF, then twice with a solution containing 150 mM NaCl, 20 mM tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, and 1 mM PMSF and subjected to kinase assays.
20. Myelin basic protein was from Sigma. ATF2 was provided by S. J. Baker and T. Curran (St. Jude Children's Research Hospital). Hexahistidine (His)-tagged c-Jun (7) and glutathione-S-transferase (GST)-catalytically inactive (K57D) *Xenopus* MAPK (34) were prepared as described. MPK2 (16), a *Xenopus* counterpart of mammalian p38, was used as a substrate for SEK1 and MKK3. SEK1 phosphorylates and activates p38 as well as SAPK at least *in vitro* (6). His-tagged catalytically inactive (K54R) p38 was prepared as described [T. Moriguchi *et al.*, *J. Biol. Chem.* **270**, 12969 (1995)]. To measure the activity to phosphorylate MBP, c-Jun, ATF2, or catalytically inactive MAPK or p38, we incubated the immune complex for 30 min at 30°C with 3  $\mu$ g of each substrate in a final volume of 25  $\mu$ l of a solution containing 20 mM tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (adenosine triphosphate) (0.3  $\mu$ Ci). The reaction was stopped by addition of Laemmli's sample buffer and boiling. After SDS-polyacrylamide gel electrophoresis (PAGE), phosphorylation of these proteins was quantified with an image analyzer (Fujix BAS2000).
21. His-tagged *Xenopus* MAPKK and SEK1 (XMEK2) and human MKK3 and MAPKK6 were bacterially expressed and purified as described [Y. Gotoh *et al.*, *Oncogene* **9**, 1891 (1994)]. To measure the activity of an immune complex, we first incubated 0.2  $\mu$ g of His-MAPKK, His-SEK1, His-MKK3, or His-MAPKK6 with the immune complex for 15 min at 30°C in a final volume of 25  $\mu$ l of a solution containing 20 mM tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 100  $\mu$ M ATP and subsequently for 7 min at 25°C with 0.3  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 3  $\mu$ g of GST-catalytically inactive MAPK (for MAPKK) or His-tagged catalytically inactive p38 (for SEK1, MKK3, and MAPKK6) in the same solution (final volume, 35  $\mu$ l). To measure the kinase activity of wild-type p38, we used His-tagged wild-type p38 and ATF2 instead of catalytically inactive p38. Samples were analyzed by SDS-PAGE and image analyzer.
22. Y. Gotoh and E. Nishida, unpublished data.
23. To avoid the possibility that constitutively expressed ASK1 might induce cell death, resulting in a failure to obtain stable transformants, we used a metallothionein-inducible promoter system. ASK1 and ASK1(K709R) cDNAs were subcloned into pMEP4 vector (Invitrogen) at convenient enzyme cleavage sites. Transfection of cDNAs was done with Transfectam (Promega) according to the manufacturer's instructions, and selection by hygromycin B was done as described [M. Saitoh *et al.*, *J. Biol. Chem.* **271**, 2769 (1996)]. Several independent colonies were cloned, and the expression of ASK1 protein was determined by immunoprecipitation (33) with antiserum to ASK1 (24). Two independent positive clones were used for the assays with essentially the same results.
24. Antiserum to ASK1 was raised against the peptide sequence TEEKGRSTEEGDCESD (amino acids 554 to 669) that was coupled to keyhole limpet hemocyanin by a glutaraldehyde method, mixed with Freund's adjuvant, and used to immunize rabbits as described (33).
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29. To measure the activity of SAPK, we subjected each cell extract to a kinase detection assay within a polyacrylamide gel (in-gel kinase assay) containing c-Jun as a substrate, as described (7). To examine the activity of p38, we immunoprecipitated p38 with polyclonal antibody to p38 (C-20, Santa Cruz) as described (19) except for the presence of 0.1% SDS during the immunoprecipitation, and the kinase activity was detected with ATF2 as a substrate.
30. H. Ichijo and K. Miyazono, unpublished data.
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32. The pcDNA3-ASK1(K709R) plasmid was transfected into Jurkat cells by DMRIE-C reagent (Life Technologies) together with pHook-1 plasmid (Invitrogen), which encodes a single-chain antibody fusion protein directed to the hapten pHox (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) and thereby allows the selective isolation of transfected cells with magnetic beads coated with pHox. ASK1(K709R)-transfected populations of cells (cotransfection efficiency was nearly 100% as determined by  $\beta$ -galactosidase staining) were isolated on pHox-coated magnetic beads with the Capture-Tec kit (Invitrogen), allowed to grow, counted, and treated with TNF- $\alpha$ . Nontransfected Jurkat cells and isolated Jurkat cells that were transfected with pHook-1, and control pcDNA3 plasmids were similarly sensitive to TNF- $\alpha$  in the DNA fragmentation assay (30).
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35. Cytoplasmic small fragmented DNA was isolated as described [K. S. Selins and J. J. Cohen, *J. Immunol.* **139**, 3199 (1987)] with minor modifications. Briefly,  $3 \times 10^6$  cells were lysed with 200  $\mu$ l of a buffer containing 20 mM tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. Cell extracts were clarified by centrifugation at 15,000g for 5 min. The lysates were incubated with proteinase K (0.2 mg/ml) and ribonuclease A (0.1 mg/ml) at 42°C for 1 hour. DNA was then purified by ethanol precipitation after phenol-chloroform extraction.
36. We thank S. J. Baker and T. Curran for ATF2; T. Maeda for TM257-H1; M. Poncz for HEL cDNA library; H. Okazaki and T. Sudo (Kirin Brewery, Japan) for oligonucleotides and advice; T. Kitagawa and C.-H. Heldin for valuable comments; A. Hanyu for technical assistance; U. Engström for preparing the synthetic peptide; and K. Saeki, T. Inage, K. Takeda, H. Nishitoh, and K. Tobiume for discussion. Supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan. H.I. and K.M. are supported by grants from Mochida Memorial Foundation for Medical and Pharmaceutical Research and Toray Scientific Foundation.

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## TECHNICAL COMMENTS

### Detecting Phases of Iron

C. S. Yoo *et al.* (1) conducted *in situ* heating experiments on Fe at pressures ( $P$ ) up to 130 GPa and conclude that (i) the  $\beta$  phase recognized by Saxena *et al.* (2) and Boehler (3) does not exist, but instead that there is an  $\epsilon'$  phase which occupies part of the stability field of the  $\gamma$  phase (FCC); (ii) only the  $\epsilon$  (HCP) phase is stable above 50 GPa; and (iii) there may be a new phase appearing above  $P$  of 110 GPa and a temperature ( $T$ ) of 3000 K. None of these conclusions is warranted on the basis of the x-ray data as presented by Yoo *et al.* (1). We have used the same experimental facilities (4) and obtained results that show evidence of transformation of  $\epsilon$  (HCP) to a DHCP structure. On the basis of our further work (5), we confirm that the DHCP phase is indeed the  $\beta$  phase and that its stability

extends beyond  $P$  of 50 GPa.

Our difficulty in accepting the results of Yoo *et al.* (1) is apparent from the data in figure 4 of their report: There is a striped area representing an  $\epsilon'$  phase of Fe in the middle of the  $\gamma$  phase field; no x-ray pattern of this  $\epsilon'$  phase is given in the report. The  $PT$  of the  $\gamma$  phase field is established from the results of several different workers on the basis of different techniques, including resistance-wire heating performed by one of the co-authors of the Yoo *et al.* (1) report. The triple point  $\gamma$ (or  $\beta$ )- $\epsilon$ -melt was located at  $76.5 \pm 4$  GPa and  $2950 \pm 100$  K by Saxena *et al.* (2) after consideration of all the available experimental data. There is no discussion of why all this data should be abandoned in favor of a study based on the use of an unstabilized laser.

One of the major problems with this type of analysis is the difficulty of interpreting the x-ray diffraction. The diffraction patterns of  $\epsilon$ -Fe (HCP) and DHCP-Fe are similar, and phase analysis of a mixture of the pressure medium and Fe phases is not a trivial task. Therefore, oxides such as  $\text{Al}_2\text{O}_3$  should not be used as the pressure medium. Corundum has many strong diffraction peaks. The laser usually heats only a part of the Fe sample, and therefore, the peaks resulting from the phase transformation are minor and closely associated with the untransformed  $\epsilon$ -iron.

We have compared the calculated pattern for mixtures of the  $\epsilon$  (HCP) and (DHCP) phases and corundum with the experimental data of Yoo *et al.* (1) (Fig. 1). The presence of a (101) DHCP-Fe reflection along with (100), (004), and (012) reflections is the diagnostic criterion for recognizing the presence of the DHCP-Fe phase because the  $\epsilon$ -Fe has only (100), (002), and (101) reflections in this range. In the presence of corundum (or Ar and LiF at  $P$  higher than 40 GPa), this diagnostic criterion does not work. Comparison of the calculated diffraction patterns (Fig. 1) shows that it is difficult to distinguish the pattern of one mixture from that of another (HCP-Fe+ $\text{Al}_2\text{O}_3$ , DHCP-Fe+ $\text{Al}_2\text{O}_3$ , and HCP-Fe+DHCP-Fe+ $\text{Al}_2\text{O}_3$ ). Reliable phase analysis requires an experimental diffraction pattern with sharp peaks [half-width (FWHM) of a reflection must be not more than 0.2 keV] from completely disoriented samples.

The diffraction patterns seen by Yoo *et al.* (1) at 50 and 96 GPa can be interpreted as an HCP-Fe+ $\text{Al}_2\text{O}_3$ , and as an HCP-Fe+DHCP-Fe+ $\text{Al}_2\text{O}_3$ , and even as a DHCP-Fe+ $\text{Al}_2\text{O}_3$  mixture. Visual asymmetry in diffraction patterns of some reflections in the study of Yoo *et al.* (1) (for example, in the interval 20 to 25 keV) and intensity correlation of reflections (in particular, in the range of 16.5 to 18.5 keV at  $P = 96$  and 125 GPa for heated and quenched Fe) may be explained in several ways, for example, by the presence of DHCP-Fe in the samples (Fig. 1).

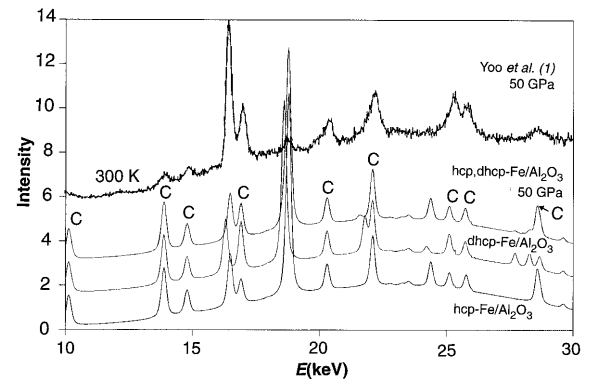
To obtain the maximum diffraction effect, Fe crystallites in the sample should be present in all possible orientations. That Yoo *et al.* (1) do not find clear DHCP-Fe reflections may be a result of the orientation of the microcrystallites in the Fe sample. Yoo *et al.* (1) note that after melting at 50 GPa, Fe crystallizes with a primary (100) orientation. However, even in the unheated Fe some peaks in the diffraction patterns were either absent or had low intensity [for example, (002), (103), and so forth]. With Fe crystallites in primary orientation, the DHCP-Fe with its closed-packed layers (001) would give practically no reflections, as may be the case in the x-ray data in the report by Yoo *et al.* (1).

In our study, the presence of a new phase  $\beta$  was detected by plotting laser power against  $T$  (2, 3). In this method, laser power is continuously increased in small steps over a large range of  $T$ . A change in slope marks the  $T$  at which a phase transformation may happen at a given pressure. Such changes were recorded independently by two different groups (2, 3) over a wide range of  $P$ . Yoo *et al.* (1) state that this might be a result of recrystallization of the sample. There is no evidence of recrystallization of the Fe foil (in our current work, we use Fe powder with the same results). We have seen recrystallization in tungsten, for example, and in that case the laser power/ $T$  relation shows a clear curvilinear pattern quite different from that of Fe. We agree with Yoo *et al.* (1) that x-ray study (in situ or quenched) of samples is the only way to obtain reliable data. However, other methods provide quick and useful information. The reflectivity (laser power/ $T$  relation) and resistivity properties belong in this category. After all, the whole question of DHCP-Fe presence started with the recognition of reflectivity changes.

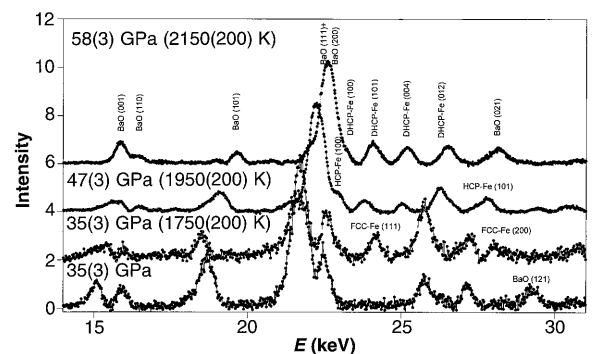
Let us assume that the uncertainty in the  $T$  measurement (1, 6) is a secondary problem.

The x-ray data on  $\epsilon'$  was not presented in the report (1). Presence of a single DHCP phase would require the complete absence of a pressure medium (corundum) in the heated area and a 100% conversion of the HCP phase to DHCP. Considering the sample geometry, the thickness of the Fe foil, the effectiveness of laser heating in the axial direction, and the radial  $T$  distribution [several hundred degrees; see discussion (7)], this is unlikely. In several tens of experiments in which we used an iron foil, we have not observed this phase. In such experiments, it is essential to find an x-ray pattern involving all three phases: namely, the medium, the untransformed HCP, and the DHCP part of Fe. Yoo *et al.* (1) heated iron foil (>20  $\mu\text{m}$  thick) that could not be totally converted from HCP-Fe to DHCP-Fe because of the inefficient heating by an unstabilized 18-W laser. The laser radiation is completely absorbed in the top few dozen nanometer thicknesses of the foil and can heat only a few micrometers at the top of the foil. We emphasize that if an x-ray pattern does not show a combination of corundum, HCP, and DHCP Fe phases, then one must be careful in interpreting it.

**Fig. 1.** Calculated and experimental x-ray pattern at 50 GPa observed by Yoo *et al.* (1). Reflection of corundum, C. We calculated x-ray patterns of mixtures HCP-Fe+ $\text{Al}_2\text{O}_3$ , DHCP-Fe+ $\text{Al}_2\text{O}_3$ , and HCP, DHCP-Fe+ $\text{Al}_2\text{O}_3$  (quantity of  $\text{Al}_2\text{O}_3 = 20\%$  in every case) using data on equations of state of HCP-Fe,  $\text{Al}_2\text{O}_3$ , and our data on lattice parameters of DHCP-Fe (4). Background was taken from figures 1 through 3 of the report by Yoo *et al.* (1), the form of reflections were described by pseudo-Voigt function, and reflections (001) of HCP- and DHCP-Fe were absent. Most intensive reflections both HCP- and DHCP-Fe are those with small indexes; for example, at  $E_d = 33.94$  keV as used by Yoo *et al.* (1), the most informative range to recognize HCP- and DHCP-Fe is that between 16 to 20 keV. This interval contains the (113) and (202) reflections of corundum. Strong (113) corundum reflection is close to the DHCP-Fe reflection of (101) at all  $P$  between 50 to 125 GPa. Intensity is in arbitrary units.



**Fig. 2.** X-ray data on Fe from different spots. All data are at room  $T$  and high  $P$ . Temperatures shown in brackets are those at which the sample was heated before quenching. Pressures are determined from the equation of state of BaO (III). Part of the Fe heated to 1750 K ( $\pm 200$ ) at 35 GPa ( $\pm 3$ ) transformed to the FCC Fe [reflections (111) and (200)]. At 47 GPa ( $\pm 3$ ) and 1950 K ( $\pm 200$ ) appear two exclusive DHCP-Fe reflections (101) and (004). They are accompanied by the (100) and (102) reflections, which could be mixtures of the DHCP- and HCP-Fe. At 58 GPa ( $\pm 3$ ) and 2150 K ( $\pm 200$ ), all HCP-Fe, which is in the form of iron powder, may have been converted to the DHCP-Fe. Intensity is in arbitrary units.



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Yoo *et al.* (1) found a new line above 2920 K at *P* higher than 110 GPa and state that “The new band was not associated with Al<sub>2</sub>O<sub>3</sub> but arose from the iron portion of the sample.” Corundum is present in all heated areas. The diffraction pattern with the new reflection at 125 GPa contains only new and corundum reflections; all Fe reflections from the  $\epsilon$  phase are absent. Perhaps Fe had melted, and the new reflection is a result of phase transition in Al<sub>2</sub>O<sub>3</sub>.

Yoo *et al.* (1) make a good point on quenched as opposed to in situ results. Some of our results (4) showing the presence of  $\beta$  phase are for in situ runs. In such cases, perhaps due to different thermal behavior of  $\beta$  and  $\epsilon$  phases, the x-ray peak positions are less resolved. Until recently, and even now, most phase diagrams are based on quenched reactions. Physically, it is quite realistic that the DHCP phase must form in *PT* field intermediate between that of HCP and FCC phases.

We conclude that the x-ray data on heated iron of Yoo *et al.* (1) do not support either the presence or absence of DHCP-Fe phase because Al<sub>2</sub>O<sub>3</sub> was used as a pressure medium (the data from experiments using Ar or LiF were not presented in the report). The corundum diffraction pattern interferes with the recognition of the DHCP-Fe pattern. The study of Saxena *et al.* (2) and a new study under way (5) show that the DHCP-Fe ( $\beta$ ) phase is a stable phase at least to 60 GPa and high *T* (Fig. 2). We achieved a complete conversion of HCP-Fe to DHCP-Fe by using iron powder in a vacuum-dried high-pressure cell. At several spots the Fe was thin enough to be thoroughly heated with a 36-W Nd-YAG stabilized (<0.1% RMS) laser.

Finally, we have recently used a resistive wire-heating method and demonstrated a reversible transformation of HCP-Fe to DHCP-Fe at *P* of 45 to 70 GPa (8).

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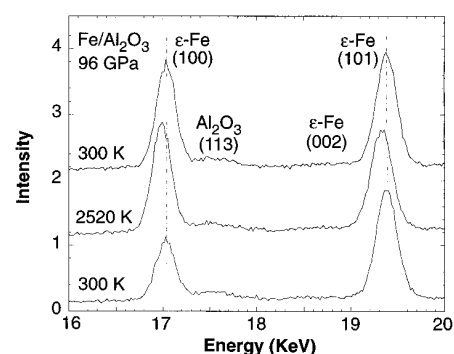
8. S. K. Saxena and L. S. Dubrovinsky, unpublished data.

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*Response:* We studied (1) the high pressure and temperature (*PT*) phase diagram of Fe with the use of an improved system integrating laser-heated diamond cell with in situ x-ray diffraction techniques. We observed the fcc-hcp-liquid triple point at  $50 \pm 10$  GPa and  $2500 \pm 200$  K and showed that the hcp-iron is the only liquid phase from 50 to 110 GPa.

Saxena and Dubrovinsky state that dhcp-iron is the liquidus phase above 50 GPa and that the dhcp-iron is the same as the  $\beta$ -phase reported previously (2–4). Both their recent and previous experiments (2–4) were indirect observations; definitive crystal structure identifications at the relevant *PT* conditions were absent. In those previous studies, high *PT* phase transitions were identified on the basis of a change in slope of laser power as a function of temperature (2, 3) or a change in visual appearance (4). However, those changes could have numerous interpretations, including ones unrelated to phase transitions—for example, they could be a result of recrystallization. Such a study does not provide information about the crystal structure of Fe. In more recent studies (2), x-ray diffraction was used for phase identification. However, that work (2) was not conducted with the integrated laser-heating and x-ray system. Reported x-ray diffraction patterns they report now and before (2) were obtained at the ambient temperature on quenched samples. Therefore, those results are not directly relevant to the crystalline phase at high *PT*, but may reflect the quench process.

The techniques, procedures, and criteria for analyzing x-ray patterns of mixed phases in diamond cells have been well established during the past two decades; the diffraction patterns for mixtures more complicated than Fe and Al<sub>2</sub>O<sub>3</sub> have been successfully resolved. We have performed more than 70 in situ x-ray diffraction measurements at simultaneous high *PT*, up to 130 GPa and 3500 K [figure 4 in (1)]. Al<sub>2</sub>O<sub>3</sub> has proven to be a good medium because its diffraction pattern is considerably weaker than that of an Fe sample; its pattern does not change in all the *PT* conditions listed in our report (1); and it forms a constant, weak, subtractable background (Fig. 1). Nevertheless, we performed control experiments with Ar, LiF, and MgO instead of Al<sub>2</sub>O<sub>3</sub> as pressure medium. In these experiments, hcp and dhcp phases (5) were readily distinguishable. Comparative study is also crucial for distinguishing alternative interpreta-



**Fig. 1.** X-ray diffraction patterns of Fe at various temperatures at 96 GPa, before (bottom), during (middle), and after (top) heating. Reversible shift and irreversible intensity of Fe diffraction lines change during the heating cycle, while the diffraction line of Al<sub>2</sub>O<sub>3</sub> remains nearly unchanged. No evidence for the dhcp phase is apparent at 2520 K and 96 GPa. Intensity is in arbitrary units.

tions. For example, at 3300 K and 125 GPa [figure 3 in (1)], the Fe diffraction pattern vanishes, new peaks appear, and the Al<sub>2</sub>O<sub>3</sub> pattern remains unaltered. Such observations are consistent with our original interpretation of a high-pressure phase of Fe or more likely a Fe-Al<sub>2</sub>O<sub>3</sub> reaction product.

Saxena and Dubrovinsky have made some in situ runs but have not reported resolving x-ray peaks. We resolved such experimental problems with an improved system, and our observations were reported accordingly (1).

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