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Regulation of c-*jun* Expression during Hypoxic and Low-Glucose Stress

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Hypoxic stress in tumor cells has been implicated in malignant progression and in the development of therapeutic resistance. We have investigated the effects of acute hypoxic exposure on regulation of the proto-oncogene c-*jun* in SiHa cells, a human squamous carcinoma cell line. Hypoxic exposure produced increased levels of c-*jun* mRNA resulting from both message stabilization and transcriptional activation. A superinduction of c-*jun* message resulted during simultaneous oxygen and glucose deprivation, with several characteristics of an induction mediated by oxidative-stress pathways. This superinduction was blocked by preincubation of cells with the glutathione precursor *N*-acetyl cysteine or with phorbol 12-myristate 13-acetate, which indicates redox control of c-*jun* expression and probable involvement of protein kinase C. By gel retardation assay, no increase in AP-1 DNA binding activity was found to be concomitant with the transcriptional activation of c-*jun*. A lack of increased DNA binding was observed for the consensus AP-1 sequence and for the two AP-1 sequence variants found within the c-Jun promoter. Additionally, hypoxic and low-glucose stress produced no activation of stably transfected AP-1 reporter sequences. Taken together, these results indicate that the transcriptional activation of c-*jun* during hypoxic and low-glucose stress involves redox control and is unlikely to be mediated by AP-1 recognition elements within the c-*jun* promoter.

Solid tumors must develop a blood supply to sustain proliferation. Because the rate of neovascularization often fails to keep pace with tumor growth, it is common for subpopulations of cells within solid tumors to experience very low oxygen and nutrient levels, as well as high levels of metabolic wastes. Hypoxic stress in tumor cells has been linked to a number of phenotypic changes fundamental to malignant progression, including DNA overreplication, gene amplification, and the development of resistance to chemotherapeutic agents (44, 45, 47, 62). In addition, recent reports have implicated hypoxia in the mediation of tumor angiogenesis (40, 52). In these studies, in situ hybridization analysis of human glioblastoma samples revealed that probable hypoxic microregions within these rapidly growing brain tumors specifically produce mRNA for vascular endothelial growth factor, a mitogen for vascular endothelial cells. Hypoxic exposure additionally was shown to produce strong inductions of vascular endothelial growth factor mRNA in several cultured cell lines (52).

Relatively little is known concerning the genetic basis of hypoxia-induced phenotypic change. Enhanced synthesis of a group of hypoxic-stress proteins (oxygen-regulated proteins, or ORPs) has been demonstrated in a variety of rodent and human tumor cell lines exposed to low levels of oxygen (25, 26, 50). The three identified ORPs, ORP 33, ORP 80, and ORP 100, are identical or very similar to heat shock protein HSP 32 (heme oxygenase-I) and the glucose-regulated proteins GRP 78 and GRP 94, respectively (38, 46, 50). Recent reports of the effects of hypoxia on tumor cell gene regulation include enhancement of epidermal growth factor synthesis, activation of heat shock transcription factor, and induction of the growth arrest and DNA damage genes *gadd45* and *gadd153* (23, 28, 41). Data concerning the regulation of genetic expression during hypoxic stress are fragmentary, but a picture in which cellular metabolism is selectively modified to enhance survival in low levels of oxygen is emerging. The molecular regulators involved in this response are unknown. We report here that hypoxic stress can strongly induce the expression of the c-*jun* proto-oncogene and, to lesser degrees, the related genes c-*fos* and *junB*.

The proto-oncogene c-*jun* plays important roles in cellular proliferation and signal transduction. Expression of c-*jun* can be induced by a variety of stimuli, including tumor promoters and growth factors, and in response to the expression of certain transforming and viral proteins (16, 29, 43, 48). The c-*jun* gene is the cellular homolog of the transforming gene of avian sarcoma virus 17 and codes for a major component of the inducible transcription regulation complex AP-1 (13, 34). AP-1 modulates gene transcription by interacting with specific variants of the DNA sequence TGA(C/G)TCA. There are AP-1 recognition sites in numerous cellular and viral genes, including early simian virus 40 sequences (for a review, see reference 58). It is notable that the promoter region of c-*jun* contains a positively acting AP-1 site, so that c-*jun* transcription can be directly stimulated by its own gene product (8). In the AP-1 complex, Jun is generally found in noncovalent association with members of the Jun and Fos protein families through coiled-coil interactions of leucine zipper domains (22, 56). The ability of human c-*jun* to participate in malignant transformation of mammalian cells was first demonstrated by Schütte and coworkers (49), who found that deregulated expression of human c-*jun* resulted in the oncogenic transformation of a rat cell line.

Our interest in the involvement of c-*jun* in hypoxia-regulated gene expression was initially sparked by reports that the DNA binding activities of Jun and Fos are modulated by reduction-oxidation of a single conserved cysteine residue in the DNA binding domains of each protein (2). Reduction of these cysteine residues was required for DNA binding. Many reductive processes are favored in hypoxic cells, including ones

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harnessed clinically to activate bioreductive hypoxic cell cytotoxins (reviewed in reference 3). Additional impetus for this work was drawn from a report that basal-level enhancement and phorbol ester induction of the mouse heme oxygenase-1 gene (ORP 33) are mediated by two high-affinity distal AP-1 sites (4). To complement ongoing studies of heme oxygenase-1 gene regulation in our laboratory, we have used SiHa cells to investigate the regulation of c-*jun* by oxygen tension. SiHa cells are a human squamous carcinoma line in which heme oxygenase-1 is strongly induced by low-oxygen exposure.

We report here that hypoxic exposure of SiHa human cells results in increased c-*jun* mRNA and protein expression and that hypoxia under low-glucose conditions produces a superinduction of c-*jun* message that is similar in several respects to the induction of c-*jun* by oxidative insult such as UV irradiation. Interestingly, no increase in either AP-1 DNA binding or transactivation of an AP-1 reporter construct was concomitant with the transcriptional activation of c-*jun*. Although the rate of synthesis of c-Jun protein increased more than fivefold during hypoxia when glucose was present, no resulting increase in AP-1 DNA binding was detected.

MATERIALS AND METHODS

Cell culture. SiHa human cervical squamous carcinoma cells (American Type Culture Collection, Rockville, Md.) were cultured in Eagle basal medium containing 10% fetal bovine serum (BME–10% FBS) at a CO_2 level of 5% (Gibco, Grand Island, N.Y.). Except where noted, SiHa cells were seeded at 1.5×10^6/100-mm-diameter culture dish on day 0 and given fresh BME–10% FBS on day 3. On day 4, 16 h before hypoxic exposure, the growth medium was replaced with BME–10% FBS containing 1 g of glucose per liter or with glucose-free BME–10% FBS. Cells were exposed to low oxygen levels within aluminum gas exchange chambers maintained at 37°C as previously described (28). Briefly, the gas within each chamber was partially replaced with a 5% CO_2–95% N_2 gas mixture repeatedly at 10-min intervals until the desired partial pressure of O_2, as monitored by an oxygen electrode (Controls Katharobic, Gulph Mills, Pa.), was obtained. Except where indicated, experiments were performed at a partial O_2 pressure of 0.01%, which required 1 h to achieve by our protocol. Respiratory consumption typically decreased O_2 partial pressures to 70 to 80% of the initial value during 5 h of hypoxic exposure. Preliminary experiments in which cells were treated with a 20% O_2–5% CO_2–75% N_2 normoxic gas mixture established that cell handling produced no effect on mRNA levels for the genes studied. Cells maintained in a 37°C CO_2 incubator were thereafter used as normoxic controls. After hypoxic exposure, the pH of cells cultured in BME–10% FBS was 7.1 to 7.2, whereas that of cells grown in glucose-free BME–10% FBS was 7.3 to 7.4. Trypan blue excludability was >93% for all treatments.

Glucose was consumed by plateau-phase normoxic SiHa cells in 100-mm-diameter plates at approximately 0.15 mg/h, as determined by hexokinase assay (Sigma Diagnostics, St. Louis, Mo.). Because cells received 10 ml of fresh medium 16 h before each experiment, the glucose concentration was typically 750 to 800 mg/liter at time zero. Glucose consumption increased during hypoxic exposure to approximately 0.74 mg/h, which would result in exhaustion of glucose after 10 to 11 h of hypoxia.

Northern RNA analysis. Total cellular RNA was isolated by guanidinium lysis and CsCl ultracentrifugation as described previously (28). Fifteen micrograms of RNA per lane was electrophoretically separated by using formaldehyde-agarose gels and blotted by capillary transfer to NitroPlus nitrocellulose membranes (Micron Separations, Westboro, Mass.). ^{32}P-labeled human c-*jun* (supplied by I. M. Verma, Salk Institute), human c-*fos* (supplied by T. Curran, Hoffmann-La Roche), human *junB* (American Type Culture Collection), and rat β-actin (supplied by C. Bitler, SRI International) cDNA probes were prepared by the random primer technique (Megaprime kit; Amersham). Where noted, a ^{32}P-end-labeled human 28S rRNA oligonucleotide probe (Clontech, Palo Alto, Calif.) was used for quantitation. Autoradiography was performed with Kodak X-AR film and Dupont Cronex Plus intensifying screens. A Lynx 4000 molecular biology workstation (Applied Imaging, Santa Clara, Calif.) was used for densitometry.

c-Jun immunoprecipitation. Metabolic labeling of aerobic samples was performed in a 37°C CO_2 incubator, whereas hypoxic samples were labeled in a 37°C humidified glove chamber maintained at 0.1 to 0.5% O_2 (Anaerobe Systems, San Jose, Calif.). Samples were labeled for 1 h in 4 ml of methionine-free BME containing 100 μCi of [^{35}S]methionine (Tran^{35}S-label; ICN, Costa Mesa, Calif.) per ml that had been degassed for several hours in the glove chamber. Glucose-free labeling medium was used for samples exposed to simultaneous oxygen and glucose deprivation. Lysates were prepared by the denaturing lysis method of Barber and Verma (11). Cells were rinsed three times in phosphate-buffered saline and lysed by addition of 0.3 ml of boiling denaturation buffer (50 mM Tris · Cl [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], 5 mM dithiothreitol). Plates were thoroughly scraped with a plastic spatula, and the viscous lysate was transferred to a 1.5-ml centrifuge tube and placed in boiling water for 10 min. The boiled lysates were then diluted with 1.2 ml of SDS-free radioimmunoprecipitation assay buffer (10 mM Tris · Cl [pH 7.4], 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 20 μg of aprotinin [Sigma] per ml, 0.25 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation at 12,000 × *g* for 10 min.

For immunoprecipitation, two 700-μl aliquots of each sample were precleared by addition of 500 μl of Immunobead reagent (goat anti-rabbit immunoglobulin) (Bio-Rad, Richmond, Calif.) and rotation overnight at 4°C. After centrifugation at 1,000 × *g* for 10 min, supernatants were transferred to fresh tubes containing 25 μl of c-*jun*/AP-1 ab1 (Oncogene Science, Uniondale, N.Y.), an affinity-purified rabbit polyclonal antibody, and rotated at 4°C for 5 h. Antigen-antibody complexes were precipitated by addition of 200 μl of Immunobead reagent, rotation for 2 h at 4°C, and centrifugation at 1,000 × *g* for 5 min. The pelleted beads were washed three times by suspension in 500 μl of ice-cold SDS-free radioimmunoprecipitation assay buffer, centrifugation at 1,000 × *g* for 5 min, and thorough drainage. To release the precipitated c-Jun, pellets were boiled for 5 min in 35 μl of SDS sample buffer (62.5 mM Tris · Cl [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS). Immunoprecipitates were resolved by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (4.5 to 12% acrylamide). After treatment with 1 M salicylic acid for 30 min, gels were dried and exposed to Kodak X-AR film at room temperature.

Message stability. The half-life of c-*jun* mRNA was determined according to the protocol of Lee et al. (30). After a 3-h incubation at 0.01% O_2, 5 μg of actinomycin D per ml was added to hypoxic samples in the glove chamber (0.1 to 0.5% O_2) described above. Total RNA was isolated beginning 10 min after actinomycin D treatment (time zero). To boost the c-*jun* signal to detectable levels in aerobic samples, cells were given fresh BME–10% FBS 90 min before time zero, as

suggested by Lee et al. (30). RNA samples were then processed for Northern blot analysis.

Nuclear runoff. Nuclei from plateau-phase SiHa cells were isolated on ice in a 4°C room by treatment with 0.5% Nonidet P-40, 10 mM Tris · Cl (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$ as described previously (9). For labeling of nascent RNA, 6×10^7 nuclei were incubated with 0.5 mM (each) ATP, TTP, and GTP, 300 μCi of [^{32}P]UTP (3,000 Ci/mmol), 20 U of Inhibit Ace (5′→3′, Boulder, Co.), and 350 μl of 2× reaction buffer (33% glycerol, 120 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0], 4 mM dithiothreitol, 50 mM ammonium sulfate, 6 mM magnesium acetate, 6 mM $MnCl_2$, 10 mM NaF, 18 mM creatine phosphate, 32 μg of creatine kinase per ml) at 30°C for 45 min with shaking (39). The reaction was terminated by digestion with 20 U of RQ1 RNase-free DNase 1 (Promega, Madison, Wis.) and 0.6 ml of HBS buffer (0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris · Cl [pH 7.4]) at 30°C for 15 min. Samples were then incubated at 37°C for 30 min with 200 μg of proteinase K (Promega) and 200 μl of SDS-Tris buffer (5% SDS, 0.5 M Tris · Cl [pH 7.4], 0.125 M EDTA) and extracted with phenol-chloroform. Isolation of RNA by filtration of trichloroacetic acid precipitates through glass fiber filters and further purification were performed as described elsewhere (9).

Nitrocellulose membrane slot blots of 400 ng each of c-*jun* cDNA, β-actin cDNA, and linearized pUC19 DNA were prepared by using a Bio-Dot SF microfiltration apparatus according to the instructions of the manufacturer (Bio-Rad). Membranes were prehybridized for 4 h in 4× Denhardt's solution (5′→3′) containing 1 μg of wheat germ tRNA (Sigma) per ml and subsequently hybridized for 48 h at 42°C with 6×10^6 cpm of nascent RNA in 50% formamide–50% hybridization solution (0.5 N NaCl, 50 mM PIPES [pH 7.0], 2 mM EDTA, 0.4% SDS) [PIPES is piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]. After hybridization, membranes were washed twice for 1 h at 45°C in buffer A (300 mM NaCl, 2 mM EDTA, 10 mM Tris · Cl [pH 7.5], 0.1% SDS) and once for 30 min at 45°C in buffer C (10 mM NaCl, 2 mM EDTA, 5 mM Tris · Cl [pH 7.5], 0.4% SDS). The blots were then washed twice briefly in buffer A, incubated for 30 min at 37°C in 10 ml of buffer A containing 100 μg of RNase A, and given a final wash for 1 h at 45°C in buffer A. Kodak X-AR film was used for autoradiography.

Transfection and reporter gene analysis. Plasmids p(AP-1)$_4$MCAT2 and pMCAT2 were provided by V. Baichwal, University of California, Berkeley. In p(AP-1)$_4$MCAT2, a chloramphenicol acetyltransferase (CAT) gene is placed downstream of a human metallothionein IIA minimal promoter and four AP-1 recognition sequences (51). pMCAT2 is the parent plasmid lacking the 68-bp AP-1 insert. Stably transfected SiHa cells were generated by electroporation with a 5:1 molar ratio of pSV2neo selection plasmid to p(AP-1)$_4$MCAT2 or pMCAT2, followed by selection with 400 μg of G418 sulfate (Gibco) per ml for 3 weeks. Populations of pooled clones were used for all experiments.

RNase protection assays were used to study AP-1-driven CAT expression in transfected SiHa cells during the first 3 h of hypoxic exposure. Tissue culture and experimental conditions were identical to those described above for the Northern analyses. Guanidinium lysis and CsCl ultracentrifugation were used for RNA isolation. To generate the protection assay riboprobe, the 287-bp *Hin*dIII-*Eco*RI fragment of pCAT-Basic (Promega) was ligated into pBluescript SK(+) phagemid (Stratagene). Transcription reactions were performed at 4°C for 15 h, and the reaction mixtures contained 100 ng of *Xho*I-cut plasmid, 15 U of T3 RNA polymerase (Promega), 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 5.5 μM UTP with 40 μCi of [^{32}P]UTP, and 500 μM (each) ATP, CTP, and GTP. Full-length transcripts were purified by electrophoresis through 5% polyacrylamide–7 M urea gels. Because the probe includes vector sequences, only 214 bp were complementary to CAT mRNA. An Ambion (Austin, Tex.) RPA II assay kit was used for the RNase protection assays. Hybridizations were performed with 2 μg of total cellular RNA and 80,000 cpm (0.2 ng) of riboprobe at 42°C for 15 h. The Ambion hybridization and RNase treatment protocols were followed. Protected fragments were identified by autoradiography of 0.35-mm-thick 5% polyacrylamide–7 M urea gels.

Gel retardation assays. Nuclear extracts were prepared from plateau-phase SiHa cells by hypotonic lysis and high-salt extraction as described previously (7). The FBS concentration in the growth medium was reduced to 0.5% 24 h before each experiment to reduce basal levels of AP-1 DNA binding. Binding reaction mixtures typically contained 5 μg of nuclear extract, 1 μg of poly(dI · dC), 5 μg of bovine serum albumin, and 1 to 10 ng of ^{32}P-labeled DNA. Reactions were performed for 20 to 30 min at room temperature in a binding buffer containing final concentrations of 20 mM HEPES (pH 7.9), 25% glycerol, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The AP-1 probe DNA was an annealed double-stranded synthetic oligonucleotide that contains the following consensus AP-1 recognition sequence:

5′-GATCGGAGCCGCAAGTGACTCAGCGCGG
3′-CCTCGGCGTTCACTGAGTCGCGCCCTAG

Where noted, double-stranded oligonucleotide probes containing the specific AP-1 recognition sequences from the c-*jun* promoter region were also used. These probes were identical to the probes identified as jun1TRE and jun2TRE by van Dam et al. (57).

Protein-DNA complexes were resolved by 5% polyacrylamide–0.5× Tris-borate-EDTA nondenaturing gel electrophoresis performed at 4°C and 15-mA constant current. Gels were subsequently dried and exposed to Kodak X-AR film.

RESULTS

Induction of c-*jun* by hypoxia in the presence and absence of glucose. Hypoxic regions of solid tumors are often severely glucose depleted because of increased glycolytic activity and inefficient vascular supply. We therefore examined the effects of hypoxia on gene expression in the presence and absence of glucose. Plateau-phase SiHa cells were preincubated for 16 h in BME–10% FBS that was either glucose free or contained 1 g of glucose per liter. The cells then were exposed to a 95% N_2–5% CO_2 atmosphere containing 0.01% residual O_2 for 2, 4, or 6 h at 37°C. As shown in Fig. 1, hypoxic exposure in the presence of glucose produced approximately fivefold inductions of c-*jun* message levels and more modest inductions of two- to threefold for c-*fos* and *junB*. No effects of glucose deprivation alone were detectable. Whereas the increases in *junB* message caused by simultaneous glucose and oxygen deprivation were indistinguishable from those caused by hypoxia alone, c-*fos* inductions were increased to approximately fourfold; >30-fold inductions were observed for c-*jun* under these conditions. Similar results were obtained with mouse EMT/Ro mammary tumor cells and with SiHa cells in BME–0.5% FBS (data not shown). No inductions were seen in identically handled SiHa samples exposed to a normoxic gas mixture containing 75% N_2, 5% CO_2, and 20% O_2.

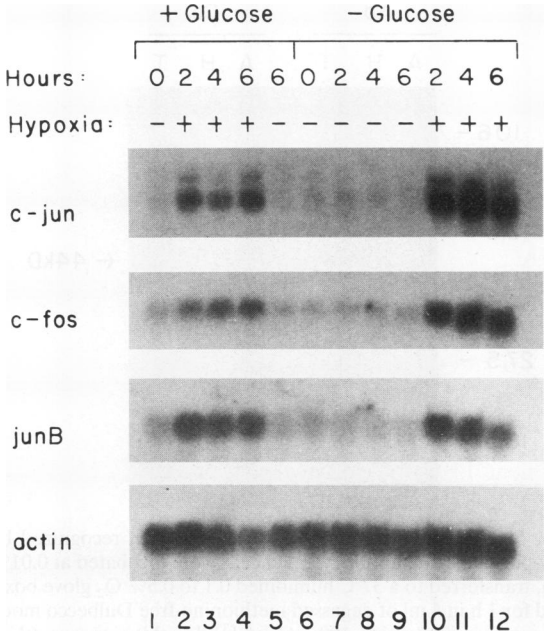

FIG. 1. Elevation of c-*jun*, c-*fos*, and *junB* mRNA levels during hypoxia. After 16 h of pretreatment with BME–10% FBS with or without glucose as indicated, SiHa cells were incubated in 0.01% O_2 for 2, 4, or 6 h. Total RNA was isolated at each time point, purified by CsCl ultracentrifugation, resolved on formaldehyde-agarose gels, blotted to nitrocellulose, and probed with ^{32}P-labeled cDNA probes for each gene. Exposure times were 16 h for c-*jun*, 48 h for c-*fos* and *junB*, and 3 h for β-actin.

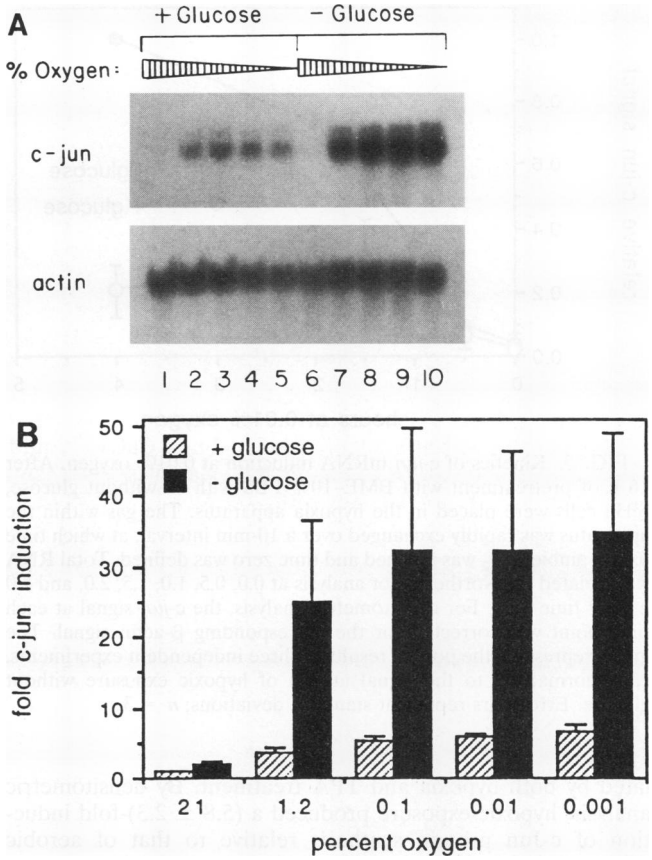

FIG. 2. Dependence of c-*jun* mRNA induction on oxygen level. (A) After 16 h of pretreatment with BME–10% FBS with or without glucose as indicated, SiHa cells were incubated for 3 h at 21% (lanes 1 and 6), 1.2% (lanes 2 and 7), 0.1% (lanes 3 and 8), 0.01% (lanes 4 and 9), or 0.001% (lanes 5 and 10) O_2. Northern blots of total RNA were prepared as described in the text and probed with ^{32}P-labeled cDNA probes for c-*jun* and β-actin. (B) Densitometric analysis of three independent experiments. Error bars represent standard deviations of fold inductions; $n = 3$.

To determine the level of oxygen required for c-*jun* induction, SiHa cells were pretreated with and without glucose as described above and incubated for 3 h at oxygen levels of 21 (atmospheric partial O_2 pressure), 1, 0.1, 0.01, and 0.001%. As shown in Fig. 2, full inductions at both glucose concentrations were achieved at 0.1% O_2. This level of hypoxia produced approximately a fivefold c-*jun* mRNA induction in the presence of glucose; glucose deprivation synergized with hypoxia to produce inductions of >30-fold. Interestingly, significant inductions were detected at the relatively high level of 1% O_2. Similar oxygen level dependencies were found for c-*fos* and *junB* (data not shown).

The induction kinetics for c-*jun* at 0.01% O_2 from three independent experiments are summarized in Fig. 3. To obtain a reasonably precise zero time point, the normal 1-h gas exchange protocol was contracted to 10 min; time zero was defined as the completion of this protocol. Rapid inductions were observed both in the presence and in the absence of glucose. A slight peak in mRNA level was consistently seen after 90 min of hypoxic exposure in samples containing glucose, followed by a decline to a steady-state level about five times greater than that in aerobic controls. Related experiments showed that the level of c-*jun* message remained elevated to 16 h of hypoxic exposure (data not shown). Simultaneous oxygen and glucose deprivation caused a strong increase of c-*jun* message that plateaued at 4 h.

The strong c-*jun* inductions that result from simultaneous oxygen and glucose deprivation are reversible, as demonstrated in Fig. 4. After a 16-h preincubation in the absence of glucose, cells were exposed to 0.01% O_2 in glucose-free medium for 6 h. Cell viability measured by trypan blue exclusion was unaffected by this treatment. Plates were then exposed to atmospheric oxygen, 1 g of glucose per liter, or both, and total RNA was extracted after recovery times of 1, 2, and 4 h. For each treatment, c-*jun* message levels showed significant movement toward basal level during the 4-h recovery period.

Induction of c-Jun protein. To correlate changes in c-*jun* transcript levels with changes in Jun protein synthesis, we pulse-labeled cells with [^{35}S]methionine and determined relative c-Jun label incorporation by resolving immunoprecipitates on SDS-PAGE gels. As a positive control, cultures were treated with 100 ng of phorbol 12-myristate 13-acetate (TPA; Sigma) per ml. The rate of total protein synthesis, measured as total label incorporation, was slightly decreased by hypoxia and slightly stimulated by TPA (expressed as a percentage of aerobic control label incorporation: hypoxia, 88% ± 13%; TPA, 109% ± 19%; $n = 3$). However, hypoxia in the absence of glucose severely inhibited protein synthesis to less than 2% of the levels in aerobic controls, precluding immunoprecipitation for this treatment. Immunoprecipitation was therefore performed only with samples exposed to hypoxia in the presence of glucose or 100 ng of TPA per ml and with aerobic controls. As shown in Fig. 5, synthesis of a 44-kDa protein recognized by an affinity-purified c-Jun antibody was stimu-

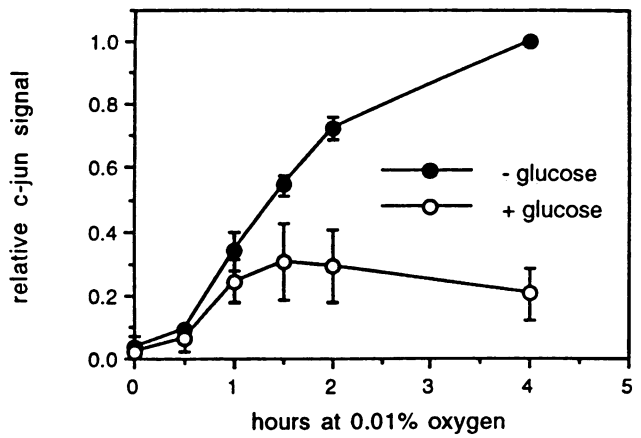

FIG. 3. Kinetics of c-*jun* mRNA induction at 0.01% oxygen. After 16 h of pretreatment with BME–10% FBS with or without glucose, SiHa cells were placed in the hypoxia apparatus. The gas within the apparatus was rapidly exchanged over a 10-min interval, at which time 0.01% ambient O_2 was reached and time zero was defined. Total RNA was isolated for Northern blot analysis at 0.0, 0.5, 1.0, 1.5, 2.0, and 4.0 h after time zero. For densitometric analysis, the c-*jun* signal at each time point was corrected for the corresponding β-actin signal. The graph represents the pooled results of three independent experiments, each normalized to the signal at 4 h of hypoxic exposure without glucose. Error bars represent standard deviations; $n = 3$.

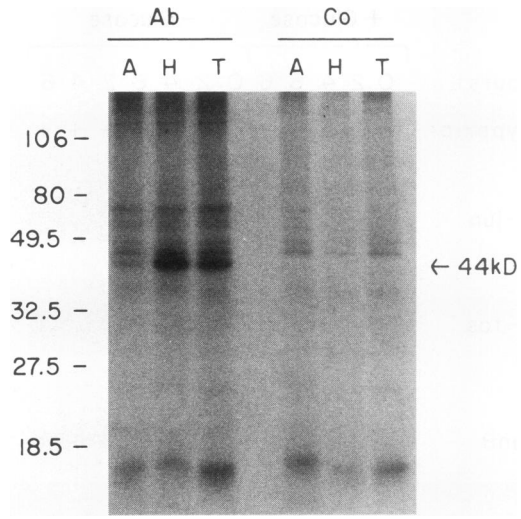

FIG. 5. Hypoxic induction of a 44-kDa protein recognized by an affinity-purified c-Jun antibody. SiHa cells were incubated at 0.01% O_2 for 1 h, transferred to a 37°C humidified 0.1 to 0.5% O_2 glove box, and labeled for 1 h in 4 ml of degassed methionine-free Dulbecco modified Eagle medium containing 100 μCi of [^{35}S]methionine per ml. As a positive control, SiHa cells were treated with 100 ng of TPA per ml for 1 h and then labeled for 1 h in a CO_2 incubator. Cells were lysed and immunoprecipitated as described in the text, using affinity-purified c-*jun*/AP-1 ab1 from Oncogene Science, resolved on discontinuous SDS-PAGE gels, and exposed to film. Ab lanes, aerobic (A), hypoxic (H), and TPA-treated (T) samples with primary antibody added; Co lanes, no primary antibody added.

lated by both hypoxia and TPA treatment. By densitometric analysis, hypoxic exposure produced a (5.8 ± 2.3)-fold induction of c-Jun protein synthesis relative to that of aerobic controls in three independent experiments.

Induction is the result of both message stabilization and transcriptional activation. Inhibitors of protein synthesis produce an accumulation of c-*jun* message in part through message stabilization (see reference 20 and references therein). Because hypoxic stress can depress total protein synthesis, we investigated the effects of hypoxia on c-*jun* message stability. Hypoxic samples were exposed to 0.01% O_2 in the presence and absence of glucose for 3 h and transferred to a 37°C glove box maintained at 0.1 to 0.5% O_2. Aerobic and hypoxic samples then were treated with 5 μg of actinomycin D per ml to block further transcription. Time zero was defined as 10 min after actinomycin D addition (30). Figure 6 shows a Northern

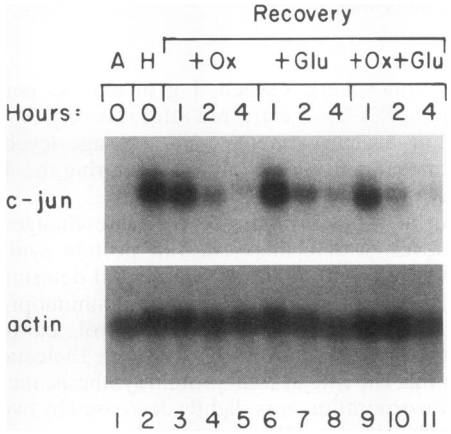

FIG. 4. Reversibility of c-*jun* mRNA induction caused by simultaneous oxygen and glucose deprivation. SiHa cells were incubated in glucose-free BME–10% FBS for 16 h and then exposed to 0.01% O_2 (lanes 2 to 11) for 6 h. Time zero was defined as completion of the initial 6 h of hypoxic exposure. At time zero, cells were exposed to atmospheric oxygen in a CO_2 incubator (+Ox), 1 g of glucose per liter in a 37°C humidified 0.1 to 0.5% O_2 glove box (+Glu), or both oxygen and glucose in a CO_2 incubator (+Ox+Glu). Samples were lysed for Northern analysis after 1, 2, and 4 h of recovery. Lane A, aerobic control at time zero; lane H, hypoxic sample at time zero.

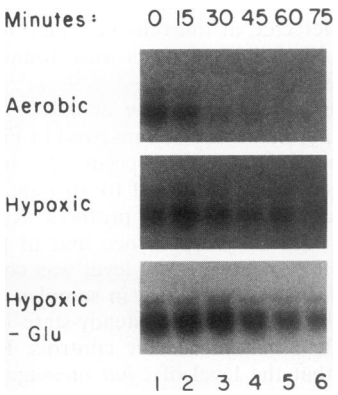

FIG. 6. Stabilization of c-*jun* mRNA by hypoxia. After pretreatment of cells with BME–10% FBS with glucose (upper two panels) or without glucose (bottom panel) for 16 h, SiHa cells were incubated at 0.01% O_2 for 3 h and transferred to a 0.1 to 0.5% O_2 glove box. Aerobic and hypoxic samples were then exposed to 5 μg of actinomycin D per ml. After a 10-min incubation, time zero was defined. Total RNA was isolated for Northern analysis at the indicated times after time zero. Aerobic samples were treated with fresh BME–10% FBS 90 min before time zero to boost c-*jun* message to a detectable level.

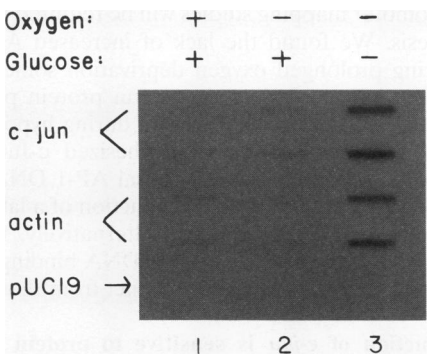

FIG. 7. Transcriptional activation of c-*jun* and β-actin during combined hypoxic and glucose stress. After 16 h of pretreatment of cells with BME–10% FBS with or without glucose as indicated, SiHa cells were incubated at 0.01% O_2 for 1.5 h. Nascent RNA was labeled with [^{32}P]UTP and isolated from nuclei as described in the text. For detection of specific transcripts, 6×10^6 cpm of each sample was hybridized to nitrocellulose slot blots (400 ng per slot) of c-*jun* cDNA, β-actin cDNA, and linearized pUC19 DNA. Each lane contains duplicate c-*jun* and β-actin slot blots and a single pUC19 slot blot.

blot of total RNA collected at 0, 15, 30, 45, 60, and 75 min after time zero. Message for c-*jun* is substantially stabilized in both hypoxic treatments. Analysis of plots of natural log (intensity) versus time from two independent experiments gave the following message half-life values: aerobic, 14.1 ± 1.2 min; hypoxic in the presence of glucose, 51 ± 13 min; and hypoxic in the absence of glucose, 46 ± 14 min. Because c-*jun* message was stabilized to the same degree in both hypoxic treatments, we reasoned that the increased message accumulation seen during hypoxic treatment in the absence of glucose was the result of increased transcriptional activity. Nuclear runoff transcription assays were performed to measure transcriptional activation directly.

Nuclei were isolated from aerobic cultures and from cultures incubated at 0.01% O_2 in the presence and absence of glucose for 1.5 h. Incorporation of [^{32}P]UTP per cell into nascent RNA was identical for aerobic samples and hypoxic samples in the presence of glucose and was slightly depressed (to 74.7% ± 5.7% of aerobic incorporation; $n = 2$) in samples deprived of both oxygen and glucose. Specific transcripts were detected by hybridization of the nascent RNA to slot blots of c-*jun* cDNA on nitrocellulose. Slot blots of linearized pUC19 DNA and β-actin cDNA were included as controls. Equal counts per minute were added to each hybridization solution, and corrections for differences in label incorporation per cell were applied during analysis of the results.

Results from the transcription assay are shown in Fig. 7. In an interesting parallel between hypoxic and oxidative stress, combined hypoxia and glucose deprivation stimulated β-actin transcription. Oxidative stress caused by an extracellular burst of active oxygen has been reported to produce a similar induction of β-actin transcription in mouse epidermal cells (17). The high basal level of β-actin present in SiHa cells partially, but not totally, masks this induction in the Northern analysis results (e.g., see Fig. 4). In terms of c-*jun* signal detected per cell, hypoxia in the presence of glucose produced a modest increase in signal (3.6 ± 1.9 times the value for aerobic controls; $n = 2$), whereas a large increase was seen for hypoxia in the absence of glucose (17.7 ± 8.2 times the value for aerobic controls). The message half-life and nuclear runoff data taken together indicate that both message stabilization and transcriptional activation are involved in the accumulation of c-*jun* mRNA during hypoxia. The two mechanisms appear to make roughly equal contributions to message accumulation during low-oxygen exposure, whereas the superinduction of c-*jun* message seen during combined hypoxic and glucose stress results from additional transcriptional activation.

No increase in AP-1-mediated transactivation or AP-1 DNA binding activity is detectable during hypoxic stress. Activation of c-*jun* transcription is commonly mediated by positively acting AP-1 sites within the c-*jun* promoter region. To test whether an elevation of AP-1-mediated transactivational activity was concomitant with the transcriptional activation of c-*jun* described above, we generated pools of SiHa cells with stably integrated AP-1 reporter sequences and performed RNase protection assays. In the reporter plasmid p(AP-1)$_4$MCAT2, CAT expression is driven by a tetrad of consensus AP-1 recognition sites (from the simian virus 40 enhancer) inserted upstream of a human metallothionein IIA minimal promoter (51). Cells were transfected with a 5:1 molar ratio of pSV2neo selection plasmid to p(AP-1)$_4$MCAT2 (or pMCAT2, the empty reporter cassette) and subjected to G418 selection. Pooled populations of G418-resistant clones were used in our experiments.

The transfected SiHa populations were untreated or pretreated for 16 h with and without glucose, incubated at 0.01% O_2 for 3 h, and lysed for RNA preparation exactly as in the Northern analyses. Aerobic samples treated with 100 ng of TPA per ml were used as positive controls. Although the TPA treatment reproducibly resulted in a protected antisense CAT mRNA fragment from cells harboring the p(AP-1)$_4$MCAT2 construct, hypoxic treatment in the presence or absence of glucose did not (Fig. 8). Therefore, no increase in transcriptional activity directed by the AP-1 consensus sequence was detected during hypoxic and low-glucose stress. The results of this assay suggest that the transcriptional activation of c-*jun* during hypoxic and low-glucose stress does not result from a net increase in consensus AP-1 transactivational activity. However, an increase in AP-1 activity specific for the AP-1 recognition sequence variants found within the c-*jun* promoter is not ruled out by these results.

Gel retardation assays were performed to determine whether a lack of AP-1 DNA binding was associated with the observed lack of AP-1 transactivation during hypoxia. The oligonucleotide probe contained a single consensus AP-1 binding sequence, TGA(C/G)TCA. SiHa cells again were preincubated for 16 h in BME–0.5% FBS containing either 1 g of glucose per liter or no glucose and then exposed to 0.01% O_2 for up to 12 h. Results of treatments for 0.5 and 6 h are illustrated in Fig. 9. At all time points to 12 h, TPA treatment increased AP-1 DNA binding, whereas hypoxic exposure in the presence or absence of glucose either had no effect or slightly reduced this activity relative to that of aerobic controls. These experiments were repeated exhaustively using various binding buffer concentrations of salt, dithiothreitol, competitor DNA, and probe DNA, including very high probe concentrations. Several running buffer pH values were also tried. No increase in consensus AP-1 DNA binding could be detected for hypoxic samples under any assay condition. Additional experiments using probes that contained the specific jun1TRE and jun2TRE AP-1 sequence variants found within the c-*jun* promoter (57) similarly detected no increase in AP-1 DNA binding during hypoxic and low-glucose stress (results not shown). Taken together with the RNase protection assay results, the observed absence of increased AP-1 DNA binding during the initial few hours of hypoxic and low-glucose stress strengthens the argument that the transcriptional activation of c-*jun* under these conditions is not mediated by AP-1 elements,

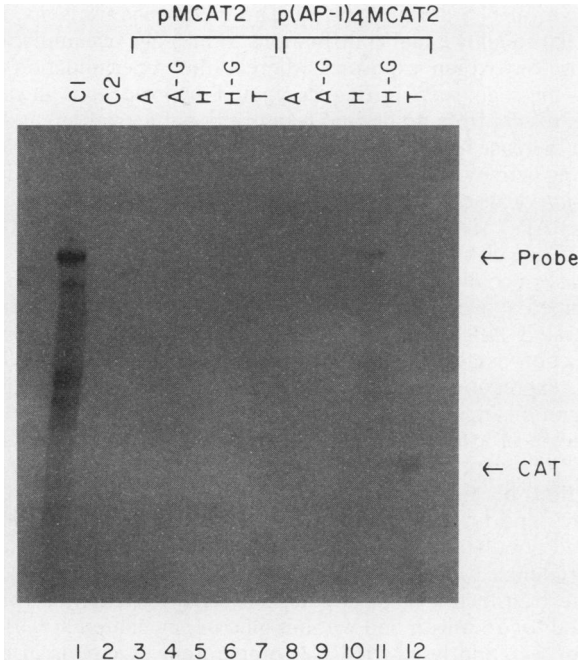

FIG. 8. RNase protection assay demonstrating activation of an AP-1-CAT reporter by TPA treatment but not by hypoxic and low-glucose stress. SiHa cells stably transfected with pMCAT2 or p(AP-1)$_4$MCAT2 as indicated were pretreated and exposed to 0.01% oxygen for 3 h as described for the Northern analysis in Fig. 2. In lanes 3 to 12, 2 μg of total cellular RNA was hybridized to 80,000 cpm (0.2 ng) of antisense CAT riboprobe. Because vector sequences were also present in the probe, only the 214-bp antisense region was protected by the CAT mRNA. Lanes: C1, undigested riboprobe; C2, no cellular RNA added; A, aerobic samples, −G, samples without glucose; H, hypoxic samples; T, TPA-treated samples.

although promoter mapping studies will be required to confirm this hypothesis. We found the lack of increased AP-1 DNA binding during prolonged oxygen deprivation somewhat surprising in light of our finding that c-Jun protein production becomes elevated greater than fivefold during hypoxia in the presence of glucose. The newly synthesized c-Jun protein produced no detectable increase in basal AP-1 DNA binding levels, which suggests the possible production of a latent c-Jun pool under these stress conditions. Alternatively, the newly synthesized c-Jun may be utilized in a DNA binding complex that is specific for DNA sequences distinct from those used in our study.

Superinduction of c-*jun* is sensitive to protein kinase C depletion and *N*-acetyl cysteine (NAC) treatment. Heme levels have been implicated in the regulation of hypoxia-responsive genes in yeast cells and in mammalian cells. Rat and human ORP 33 (heme oxygenase-1) expression can be stimulated by heme compounds (33, 61). Heme represses the expression of the anaerobic gene *ANB1* in *Saccharomyces cerevisiae*, whose product interestingly shows extensive homology to the human and rabbit translation initiation factor eIF-4D (32, 35). To test the possibility that heme could similarly repress anaerobic induction of c-*jun*, we pretreated SiHa cultures for 16 h with 30 μM hemin in the presence and absence of glucose and exposed them to 0.01% O_2 for 3 h. Hemin exposure produced no effect on aerobic, hypoxia-induced, or TPA-induced levels of c-*jun* mRNA (data not shown). In contrast, cycloheximide treatment strongly induced c-*jun* message levels in SiHa cells, which precluded the use of cycloheximide to study the mechanism of hypoxic c-*jun* induction.

Protein kinase C mediates the induction of c-*jun* by various stimuli, including TPA (29). To investigate protein kinase C involvement in the hypoxic induction of c-*jun*, we depleted protein kinase C activity by treatment of cells with 100 ng of

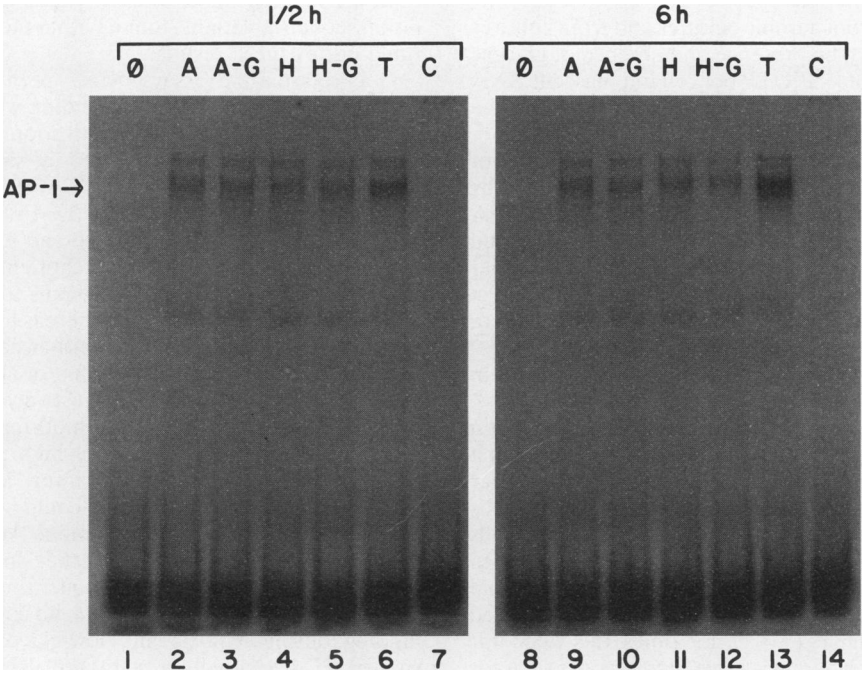

FIG. 9. Hypoxic exposure induces no AP-1 DNA binding. After pretreatment of cells with BME–0.5% FBS or glucose-free (−G) BME–0.5% FBS for 16 h, SiHa cells were incubated at 0.01% O_2 for the indicated times. Gel retardation assays were performed with a consensus sequence AP-1 probe as described in the text. Lanes 1 and 8, no cell extract added; lanes 7 and 14, duplicates of lanes 4 and 11, respectively, but containing a 50-fold excess of unlabeled probe DNA. A, aerobic samples; H, hypoxic samples; T, TPA-treated samples; C, competitor.

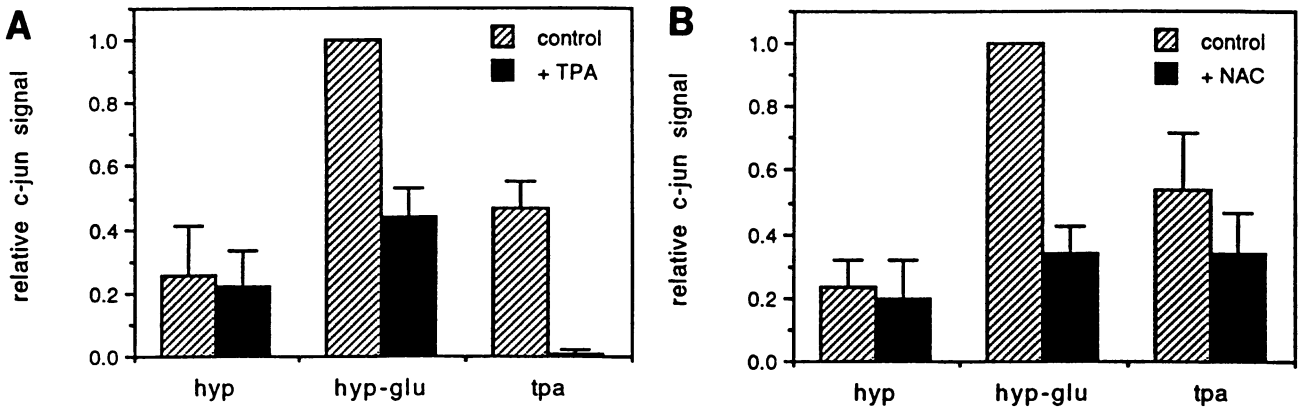

FIG. 10. Reduction of c-*jun* message accumulation during combined hypoxic and glucose stress by pretreatment of cells with TPA or NAC. (A) SiHa cells were incubated with 100 ng of TPA per ml or with dimethyl sulfoxide carrier for 40 h and then exposed to 0.01% O_2 for 3 h. Cells were given fresh BME–10% FBS with or without glucose, along with fresh TPA or dimethyl sulfoxide, 16 h before hypoxic exposure. (B) After incubation in BME–10% FBS with or without glucose for 15 h, cells were treated with 40 mM NAC for 1 h and exposed to 0.01% O_2 for 3 h. Northern blots of total RNA were prepared as described in the text and probed with ^{32}P-labeled probes for c-*jun* and 28S rRNA. For densitometric analysis, the c-*jun* signal for each treatment was corrected for the corresponding 28S signal. The results of two (TPA) or three (NAC) independent experiments are represented, each normalized to the control induction (hypoxia minus glucose). Error bars represent standard deviations.

TPA per ml for 40 h before hypoxic exposure (reference 27 and references therein). As shown in Fig. 10A, this treatment eliminated c-*jun* responsiveness to an additional TPA challenge, which indicates depletion of protein kinase C activity. Transcripts for c-*jun* were still inducible by exposure to 0.01% O_2 for 3 h. Although the level of induction caused by hypoxia in the presence of glucose was unaffected by TPA pretreatment, the induction produced by simultaneous oxygen and glucose deprivation was attenuated by about half (to 0.44 ± 0.09 of the nonpretreated value; $n = 2$). This result may reflect an involvement of protein kinase C in the large transcription activation of c-*jun* seen during combined hypoxic and glucose stress.

The induction of c-*jun* message caused by oxidative stress from UV irradiation can be blocked by preincubation of cells with NAC (19). NAC can readily enter cells. There it is converted to glutathione (GSH), which participates in the intracellular GSH redox buffering system (36). To further probe the parallels between hypoxic and oxidative stress, we preincubated cells with NAC for 1 h before hypoxic exposure. As shown in Fig. 10B, NAC had no statistically significant effect on the induction of c-*jun* by hypoxia in the presence of glucose or by TPA. However, the magnitude of the induction caused by simultaneous oxygen and glucose deprivation was decreased in the presence of NAC to approximately that seen for hypoxic stress alone. NAC treatment had no effect on aerobic c-*jun* message levels, in contrast to the results of studies in which HeLa cells were used (37). These results provide an additional link between the superinduction of c-*jun* caused by oxidative stress and that caused by combined oxygen and glucose deprivation (see Discussion). The connection between the TPA and NAC results is not clear, but it is interesting that tyrosine kinases play a central role in the mammalian UV response (19).

DISCUSSION

We have shown that both message stabilization and transcriptional activation contribute to the increased levels of c-*jun* mRNA found during hypoxic stress. Combined oxygen and glucose deprivation produces superinduced c-*jun* mRNA levels in a manner that resembles an oxidative-stress response in several respects. First, the c-*jun* induction characteristics closely parallel those observed in HeLa cells exposed to 260-nm irradiation (18). In that study, UV exposure rapidly induced mRNA for c-*jun* 55-fold, whereas c-*fos* and *junB* message levels showed more modest increases. UV was a stronger inducer of c-*jun* than TPA, but both c-*fos* and *junB* were more responsive to TPA than to UV. These same findings hold true during the cellular response to simultaneous hypoxic and glucose stress. Second, c-*jun* and β-actin show concomitant transcriptional activation, as has been demonstrated in mouse JB6 cells exposed to an extracellular burst of active oxygen generated by xanthine-xanthine oxidase treatment (17). Third, the superinduction of c-*jun* mRNA is blocked by preincubation of cells with the GSH precursor NAC. NAC similarly blocks the induction of c-*jun* mRNA by UV (19). These three parallels with oxidative stress may be related to a depletion of the intracellular reduced GSH pool during combined oxygen and glucose deprivation. Presumably, production of NADPH via the pentose phosphate pathway is strongly suppressed by these starvation conditions. Because GSH turnover is fueled by NADPH, the redox balance maintained by the intracellular GSH redox buffer system may undergo an oxidative shift. We are preparing to test this hypothesis by direct measurement of intracellular reduced and oxidized GSH.

In contrast to studies with UV-irradiated cells, however, we find no evidence that the transcriptional activation of c-*jun* observed during hypoxic and low-glucose stress is mediated by AP-1 response elements. AP-1 DNA binding and transactivation of an AP-1-CAT reporter both remain at basal levels during the first few hours of stress, whereas TPA treatment stimulates these activities. In UV-irradiated HeLa cells, AP-1 elements clearly have been shown to mediate the transcriptional response of the c-*jun* gene (18, 54). The identification of hypoxic response elements within the promoter regions of c-*jun* and other ORPs is a current focus in our laboratory.

During more prolonged hypoxic exposure in the presence of glucose, we have found that elevated levels of c-Jun protein expression fail to result in increased AP-1 DNA binding. The DNA binding activity of Jun is subject to multiple regulatory mechanisms involving dimerization partner, phosphorylation-dephosphorylation, and reduction-oxidation. Jun can form

heterodimers with at least nine other leucine zipper proteins (10). The different heterodimers generally exhibit distinct characteristics of DNA interaction. For example, Jun/ATF-2 heterodimers have a much greater binding affinity for CRE sequences than for AP-1 sites (24). Under conditions of low oxygen, c-*jun* is induced to a greater degree than c-*fos*. Selective expression of this type would be an effective mechanism for the cellular production of Jun protein under conditions in which it can bind with dimerization partners other than Fos, possibly producing heterodimers that preferentially interact with sequences distinct from AP-1 recognition sites.

The undetectable DNA binding activity of c-Jun produced during hypoxic stress may also be related to its phosphorylation state. The DNA binding and transactivational activities of c-Jun are modulated by at least five sites of serine and threonine phosphorylation (12, 15, 21, 42, 53). Three phosphorylation sites located adjacent to the DNA binding domain reportedly can modulate DNA binding directly. Two or three of these sites are phosphorylated in resting fibroblasts and epithelial cells, resulting in inhibition of DNA binding activity (15). Treatment with phorbol esters leads to dephosphorylation of at least one of these residues and is coincident with an increase in DNA binding. Evidence that casein kinase II may be responsible for in vivo negative regulation of c-Jun DNA binding by this mechanism has been presented elsewhere (31). Regulation of AP-1 DNA binding by phosphorylation is likely to involve additional complexities, judging by the conflicting results being produced by different laboratories (10). The phosphorylation state of Jun under hypoxic stress has not yet been determined.

The DNA binding activity of Jun-Jun homodimers and Jun-Fos heterodimers can also be regulated by reduction-oxidation of a cysteine residue within the DNA binding domains of each protein. Reduction of these cysteines is necessary for high-affinity DNA binding (1, 2). A 37-kDa nuclear factor exhibiting redox activity for these cysteines has been isolated from HeLa cells and cloned (59). This protein, Ref-1, is identical to the human apurinic-apyrimidinic endonuclease HAP1 (60). Ref-1 is therefore a bifunctional protein possessing both AP-1 redox and DNA repair activities. If the critical cysteines are not maintained in a reduced state, the DNA binding activity of Jun protein produced during hypoxia will be inhibited.

Further studies will be needed to determine the phenotypic consequences of elevated levels of c-Jun expression in hypoxic cells. Like many components of the cellular pathways through which growth signals are transduced, c-Jun has oncogenic potential that can be activated by structural changes or by aberrant expression. Several studies support a conclusion that c-*jun* overexpression in mammalian cells is at best weakly oncogenic but can cooperate with other growth signals to produce transformation (see reference 58). Recent studies of cells derived from transgenic mice that harbor the bovine papillomavirus type 1 genome demonstrate that elevation of the levels of c-*jun* and *junB* expression can cause progression of fibromastoses to fibrosarcomas (14). Interestingly, normal rat fibroblasts exposed to anoxic conditions undergo a stepwise series of alterations that parallel certain changes associated with malignancy (55). Transcriptional activation of VL30 sequences is the initial response (6), followed by secretion of the cancer-associated metalloproteinases cathepsin D and cathepsin L (5) and by the induction of an endonuclease activity that has been proposed as a potential cause of genomic instability in cancer cells (55). The role of c-*jun* expression in hypoxia-induced phenotypic change has not yet been investigated in normal cells or in tumor cells. Such studies are likely to provide insight into the mechanisms by which microenvironments within solid tumors influence malignant progression and therapeutic resistance.

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