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Intracellular Responses of Antibody-Producing H69K-NGD Transfectoma Subjected to Hyperosmotic Pressure

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Abstract When subjected to hyperosmotic pressure by NaCl addition, H69K-NGD transfectoma, like KR12H-2 transfectoma, displayed decreased specific growth rate (μ) and increased specific antibody productivity (q_{Ab}): Elevation of medium osmolality from 280 mOsm/kg to 415 mOsm/kg decreased μ by 79% in batch cultures of H69K-NGD transfectoma, while it increased q_{Ab} by 103%. However, unlike KR12H-2 tranfectoma, enhanced q_{Ab} of H69K-NGD transfectoma at hyperosmolalities was not due to elevated levels of Ig mRNAs. In hyperosmotic cultures of H69K-NGD transfectoma, heavy-chain mRNA per cell was not enhanced with increasing osmolality. Hyperosmotic pressure was found to preferentially enhance immunoglobulin (Ig) translation rates of H69K-NGD transfectoma. However, under hyperosmotic pressure, the translation rate of Ig polypeptides was not enhanced as much as q_{Ab}. This result suggests that hyperosmotic pressure also influences the post-translational process. Taken together, the results obtained show that intracellular response of transfectomas to hyperosmotic pressure, in regard to the main intracellular steps of the antibody secretory pathway, is cell-line dependent.

Key words: Antibody, hyperosmotic pressure, transfectoma

The increasing demand for large quantities of monoclonal antibody for *in vitro* (e.g., diagnostic or immunoprecipitation) and for *in vivo* (e.g., immunotherapy and *in vivo* imaging) application necessitates optimization of existing production methods and, possibly, the development of new ones [1, 6, 10]. Strategies to enhance antibody yield extensively involve the medium formulation [3], modes of cell propagation [17], and the design of high-performance reactor configuration [12]. Most of these have been focused on bioreactor design and operation scheme to achieve the high cell concentration. Recently, hyperosmotic pressure, which can be induced by addition of cheap salts or sugars, has been suggested as an economical solution to increase the specific antibody productivity (q_{Ab}) in hybridoma [10, 14, 15], transfectroma [11], and Chinese hamster ovary (CHO) cell culture [18]. However, the enhanced q_{Ab} in hyperosmolar batch cultures does not necessarily result in a substantial increase of the final antibody concentration, because a hyperosmotic culture condition suppresses cell growth [10, 14, 11, 18].

To overcome this drawback, extensive efforts have been made regarding cell cultures. As a result, several strategies, such as adaptation of cells to hyperosmotic pressure [14], use of osmoprotectants in media [16, 18], overexpression of antiapoptotic gene [7], and two-stage culture [17] have successfully been applied for improved antibody production. In contrast, studies on intracellular responses of transfectomas to hyperosmotic pressure have not been fully substantiated. The detailed mechanisms of enhanced q_{Ab} have not yet been clearly understood at the basic cellular levels, although it could eventually lead to a better insight into possible environmental or genetic manipulation approaches for enhancing q_{Ab}. Furthermore, in order to accept generality of the effect of hyperosmotic pressure on q_{Ab}, more cell lines need to be tested.

In order to determine how hyperosmotic pressure influences any of the main intracellular steps of the antibody secretory pathway, such as transcription, translation, and secretion, we investigated the intracellular responses of H69K-NGD transfectoma expressing a mouse/human chimeric antibody to hyperosmotic pressure, generated by NaCl addition.

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MATERIALS AND METHODS

Cell Line

The cell line used in this study was H69K-NGD transfectoma expressing a chimeric antibody against the pre-S2 surface antigen of hepatitis B virus (HBV). This transfectoma was made by sequentially electroporating the linearized heavy and light chain-specific expression plasmids (pHS2-neo and pLS-hygro, respectively) into Sp2/0-Ag14 hybridoma (ATCC CRL 1581) [5].

Culture Medium and Cell Culture

The medium used for culture maintenance was Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO, U.S.A.) supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY, U.S.A.). No antibiotics were added to the medium. The cells were maintained in T-flasks (Bellco Glass, Vineland, NJ, U.S.A.) at 37°C in a humidified 5% CO_2 incubator.

Hyperosmolar culture media with various osmolalities were prepared by addition of NaCl into the standard medium (IMDM supplemented with 5% FBS, 280 mOsm/kg). The osmolalities of the three different hyperosmolar media prepared were 325, 370, and 415 mOsm/kg.

For batch cultures, cells growing exponentially in the standard medium were inoculated into T-75 cm^2 flasks (Bellco) containing media with various osmolalities. The initial cell concentration was approximately 2×10^5 cells/ml, and cells were cultured three separate times in a humidified 5% CO_2 incubator.

Cell Concentration, Osmolality, and Antibody Assay

Samples for cell counts and antibody assay were taken twice a day. Viable cell concentration and viability were determined by the trypan blue dye exclusion method using a hemocytometer. The samples were then centrifuged at 200 ×*g* for 10 min, and the supernatants were kept frozen at −80°C for later analysis.

The secreted chimeric antibody with anti-pre-S2 specificity was quantified using an enzyme-linked immunosorbent assay (ELISA) as described previously [3, 8]. Briefly, the pre-S2 peptide (Sigma) was used to coat the microtiter plate wells at a concentration of 2.5 μg/ml. Purified chimeric antibody was used as a standard. Peroxidase-labeled goat anti-human IgG (Fc specific, Sigma) was used as an enzyme-antibody conjugate. The q_{Ab} was based on the data obtained during the exponential phase of growth and was evaluated as described earlier [2, 10]. Osmolality was measured using an osmometer (Automatic semi-micro osmometer, model A0300, Knauer, Berlin).

Dry Cell Weight (DCW) and Total Cellular Protein Content

For the determination of DCW, the cells in the exponential growth phase were harvested by centrifugation and washed twice with cold phosphate-buffered saline (PBS). Approximately 1.6×10^7 cells were then resuspended in 1 ml of deionized water, transferred to pre-weighed foil dish, and dried overnight at 105°C before weighing on a scale.

For the determination of total cellular protein content, approximately 8×10^6 cells were lysed in 0.5 ml of lysis buffer [1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris-Cl (pH 7.4), 0.02% sodium azide, and 2 mM phenylmethylsulfonyl fluoride] on ice for 1 h, and the resulting lysate was centrifuged at 10,000 ×*g* for 15 min to remove cell debris. The supernatant was assayed for protein by the bicinchoninic acid (BCA) protein assay (Sigma) with bovine serum albumin (BSA, Gibco) as a standard.

Western Blot Analysis

Proteins were resolved on a 10% SDS-polyacrylamide gels using the buffer system described by Laemmli [9]. Following electrophoresis, the separated proteins were blotted onto nitrocellulose membrane (Hoefer, San Fernando, CA, U.S.A.) using a Hoefer Trans-Blot apparatus. Subsequently, the nitrocellulose membrane was blocked with TBS containing 3% BSA for 2 h at room temperature. The membrane was rinsed twice with TBS containing 0.05% Tween 20 (TBST), and then incubated for 30 min at room temperature with anti-human IgG (H+L) conjugated with horse-radish peroxidase at 1:2,000 (Promega, Madison, WI, U.S.A.). Detection of the antigen-antibody complex was performed with enhanced chemiluminescence (ECL, Amersham, U.K.). For quantification, several exposures of samples were scanned, and values in a linear range of the film were analyzed.

Ribonuclease Protection Assay

Total cytoplasmic RNA was isolated from the cell pellets [19]. Hybridization was performed by mixing 20 μg of total cellular RNA and 5×10^5 cpm of the antisense RNA probe in 30 μl of hybridization buffer containing 80% formamide, 1 mM EDTA, 40 mM 1,4-piperazine-diethane sulfonic acid (PIPES, pH 6.4), and 0.2 M sodium acetate. The RNA mixture was denatured at 85°C for 5 min and then allowed to reanneal overnight at 45°C. To this hybridized mixture, 5 to 10 units of RNase ONE (Promega) were added, and unprotected RNA was digested at 20°C for 1 h. The protected RNA-RNA hybrids were extracted, precipitated, and electrophoresed on an 8% denaturing polyacrylamide gel.

For the preparation of the heavy and light chain specific antisense RNA probes, 234-bp of a *Acc*I-*Hin*dIII fragment of pMH and 134-bp of a *Kpn*I fragment of pMK were subcloned into pBluescript SK(+) and pGEM-3Zf(+), respectively [4]. The resulting plasmids were then digested with *Xba*I and *Hin*dIII, respectively, and were used as templates for *in vitro* transcription. The *in vitro* transcription

reaction was performed using T7 RNA polymerase (Boerhinger Mannheim) and [α-^{32}P]CTP at 37°C, according to the protocol provided by the supplier. RNase-free DNase I (Boerhinger Mannheim) was added to the reaction mixture, and the reaction was continued for another 15 min. The radioactive RNA probe was extracted, precipitated, and dissolved in the hybridization buffer, and the specific radioactivity was then determined using a Liquid Scintillation Counter (LS 6000LL, Beckman, Fullerton, CA, U.S.A.).

Biosynthetic Labeling and Immunoprecipitation

For the metabolic labeling of cellular proteins, exponentially growing cells (5×10^6 cells) were labeled for 30 min in the medium containing 200 μCi of [^{35}S]methionine after 1 h starvation in methionine-free medium. Cell lysates were prepared in a lysis buffer (0.15 M NaCl, 50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.02% sodium azide, and 2 mM phenylmethylsulfonyl fluoride) on ice. Aliquots of cell lysates containing 5×10^6 trichloroacetic acid-precipitable cpm or supernatant equivalent to that cpm were applied to immunoprecipitation. Labeled samples were diluted in 0.5 ml of TBS containing 0.1% bovine hemoglobin and then immunoprecipitated with affinity purified goat antibodies against human γ chain and κ chain in combination with protein G sepharose. Sepharose-bound immune complexes were washed four times with 0.05% Nonidet P-40 in TBS and once with TBS. Bound antigens were eluted from beads by boiling in SDS sample buffer with or without 3% 2-mercaptoethanol. Purified protein was analyzed on a 10% polyacrylamide gels using the Laemmli buffer system [9]. Gels were then stained with Coomassie brilliant blue. To quantify radioactivity incorporated into the proteins, the band intensities on autoradiograms were determined using an ultrascan laser densitometer (Model N0. 2202; LKB Instruments, Gaithersburg, MD, U.S.A.).

RESULTS

Cell Growth and Antibody Production

To determine the effect of hyperosmotic pressure on H69K-NGD transfectoma in regard to cell growth and antibody production, batch cultures with various osmolalities were performed over a period of 5 d.

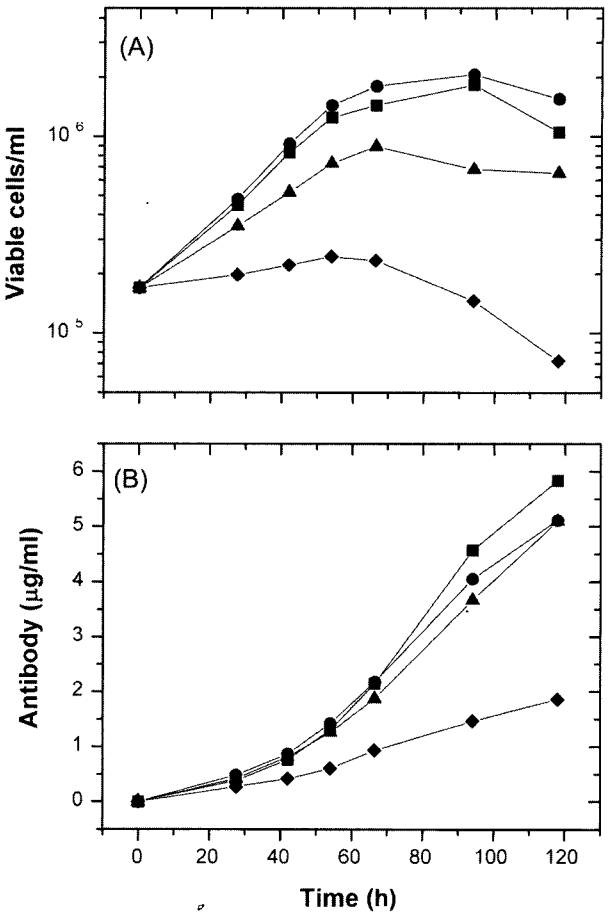

Fig. 1. Batch cultures of H69K-NGD transfectoma at various osmolalities.
Viable cell concentration (A) and antibody concentration (B). (●) 280 mOsm/kg; (■) 325 mOsm/kg; (▲) 370 mOsm/kg; (◆) 415 mOsm/kg.

Figure 1 shows typical cell growth and antibody production profiles during batch cultures. The osmolalities did not change significantly during the culture (data not shown). When subjected to hyperosmotic pressure, however, cell growth was depressed (Fig. 1A). When the osmolality was increased from 280 to 415 mOsm/kg, the μ decreased by approximately 79%. On the other hand, the highest antibody concentration was obtained at elevated osmolality (325 mOsm/kg), despite the depressed cell growth (Fig. 1B). This result implies that q_{Ab} was significantly enhanced at hyperosmolalities. When the osmolality was increased

Table 1. Effect of hyperosmotic pressure on μ and q_{Ab} of H69K-NGD.[a]

Osmolality (mOsm/kg)	μ (h^{-1})	Relative μ	q_{Ab} (μg/10^6 cells/day)	Relative q_{Ab}
280	0.0397±0.0021	1	0.8049±0.0213	1
325	0.0360±0.0011	0.93	1.0911±0.0493	1.36
370	0.0266±0.0019	0.69	1.5366±0.2829	1.91
415	0.0079±0.0013	0.21	1.6342±0.1985	2.03

[a]Means±SD. This experiment was repeated three separate times.

Table 2. Effect of hyperosmotic pressure on DCW and total protein content of H69K-NGD.[a]

Osmolality (mOsm/kg)	DCW (μg/10^6 cells)	Relative DCW	Total cellular protein (μg/10^6 cells)	Relative total protein content
280	326.9±2.7	1	166.2±3.9	1
325	370.9±5.3	1.13	189.2±2.8	1.14
370	441.2±11.9	1.35	207.5±2.2	1.25
415	506.4±8.1	1.55	231.5±3.7	1.39

[a]Means±SD. This experiment was repeated three separate times.

from 280 to 415 mOsm/kg, the q_{Ab} was increased by 103%. The μ and q_{Ab} at various osmolalities are summarized in Table 1.

DCW and Total Cellular Protein Content

To determine DCW and total cellular protein content, exponentially growing cells were sampled from batch cultures at various osmolalities.

When subjected to hyperosmotic pressure, DCW was increased: The DCW at 415 mOsm/kg was 506.4±8.1 μg/10^6 cells (average±standard deviation), which is 55% higher than that at 280 mOsm/kg. The changes in total cellular protein due to hyperosmotic pressure were similar to those in DCW. Thus, the ratio of total cellular protein content to DCW remained almost constant at 0.46–0.51. The DCW and total cellular content at various osmolalities are summarized in Table 2.

Intracellular Levels of Heavy and Light Chain Polypeptides

The intracellular heavy and light chain polypeptides contents were assessed by Western blot analysis of cell lysates used for protein assay. An equal amount of protein sample at each osmolality was separated on 10% polyacrylamide gel and blotted onto nitrocellulose membrane (Fig. 2A). The band intensity of heavy and light chain polypeptides shown in Fig. 2A was quantified by densitometric analysis (Fig. 2B). When the osmolality was increased from 280 to 415 mOsm/kg, approximately two-fold increase in the level of light chain was observed. In contrast, the level of heavy chain decreased by about 30%. When the immunoglobulin (Ig) polypeptides contents were calculated on a per-cell basis, the increase of light-chain polypeptide was further intensified at elevated osmolalities (Fig. 3). The heavy-chain polypeptide content on a per-cell basis remained almost constant regardless of osmolalities used, suggesting

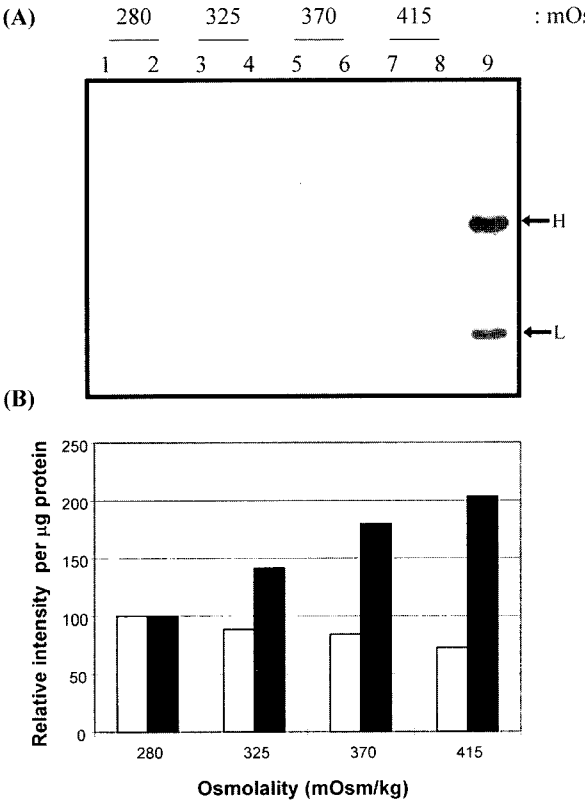

Fig. 2. Effect of hyperosmotic pressure on intracellular heavy and light chain polypeptides contents; (A) Western blot detection of heavy (H) and light (L) chains.
Cell lysates from independent cell cultures at the indicated osmolality were loaded on lanes 1–8. Purified H69K-NGD antibody was loaded on lane 9. (B) Densitometric analysis of intracellular heavy (■) and light (□) chain levels. The band intensity at the indicated osmolalities was normalized by that at 280 mOsm/kg.

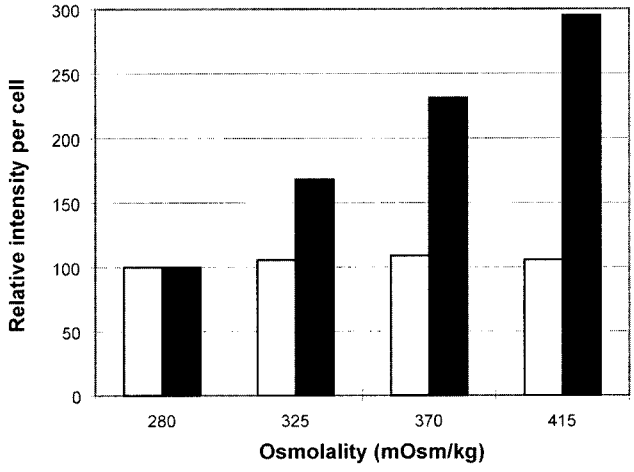

Fig. 3. Effect of hyperosmotic pressure on intracellular heavy (■) and light (□) chain polypeptides levels on a per-cell basis.
The intracellular heavy and light chain polypeptides contents at the indicated osmolalities were normalized by those at 280 mOsm/kg.

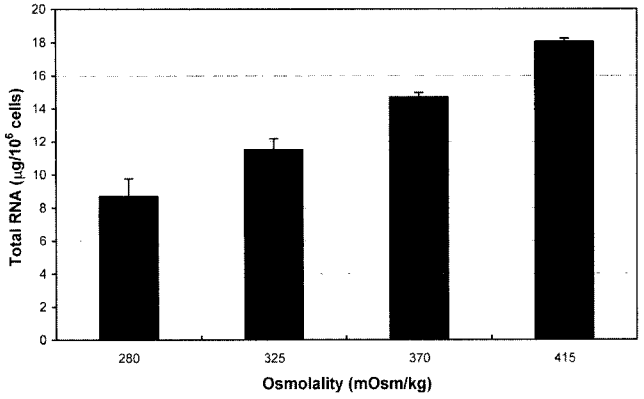

Fig. 4. Effect of hyperosmotic pressure on total RNA content.
The concentration of RNA in each sample was determined by measuring the optical density (OD) of the sample at 260 nm.

that the increasing cellular protein content at elevated osmolality does not involve increase of heavy-chain content.

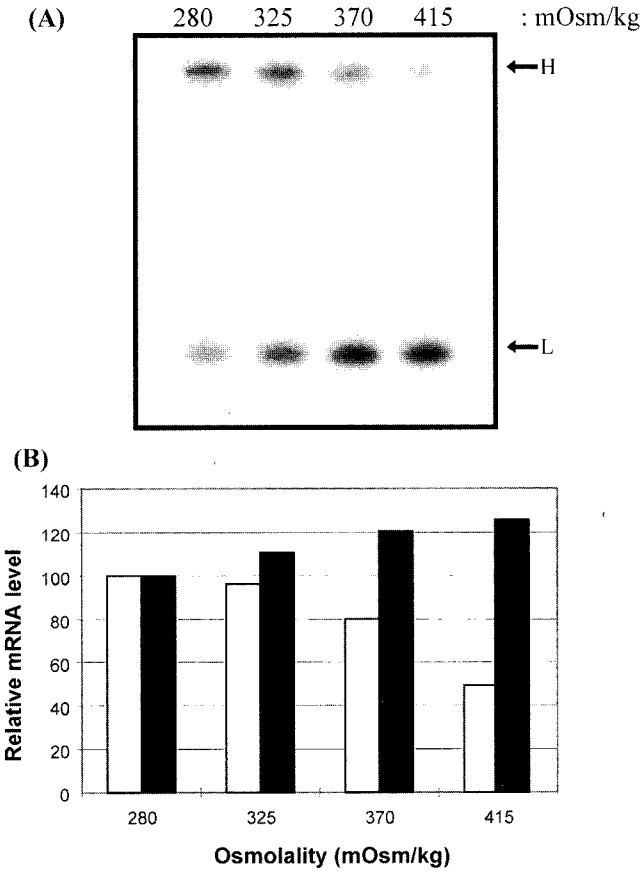

Fig. 5. Effect of hyperosmotic pressure on heavy and light chain mRNAs.
(A) Ribonuclease protection assay of heavy (H) and light (L) chain-specific mRNAs of H69K-NGD during batch cultures at the indicated osmolalities. (B) Relative heavy (■) and light (□) chain mRNA content per μg RNA at indicated osmolalities. The band intensity at the indicated osmolalities was normalized by that at 280 mOsm/kg.

Total Cellular RNA and Heavy and Light Chain mRNA Levels

To understand the effect of hyperosmotic pressure on q_{Ab} at the basic cellular levels, possible changes in total cellular RNA and Ig mRNA levels caused by hyperosmotic pressure were examined with exponentially growing cells at various osmolalities.

Figure 4 shows the total cellular RNA content at various osmolalities. No gross degradation of RNA was detected by an electrophoretic method utilizing formaldehyde (data not shown). Total cellular RNA per cell increased at higher osmolalities: at 415 mOsm/kg. The total cellular RNA per cell increased by approximately 105% compared with that at 280 mOsm/kg.

Figure 5 shows the relative Ig mRNAs at various osmolalities, determined by ribonuclease protection assay of total cellular RNA to radioactively labeled antisense RNA probes. When subjected to hyperosmotic pressure, light-chain mRNAs in total cytoplasmic RNA were increased. In contrast, heavy-chain mRNAs decreased with increasing osmolality, suggesting that the decreased transcription level of heavy chain is partly responsible for the decreased level of heavy-chain polypeptide at elevated osmolalities, as determined by Western blot assay (Fig. 2).

The ratio of the light-chain mRNA to heavy-chain mRNA was calculated by correcting the ratio of the respective protected signals for the corresponding mRNAs, the specific activity of the each RNA probe, and the length of the protected probes. This ratio was found to increase from 1.7 to 4.3 as medium osmolality was elevated from 280 to 415 mOsm/kg (Fig. 6).

Heavy and Light Chain mRNAs-Specific Translation Rates

To investigate the synthesis of Ig polypeptides, biosynthetic labeling was performed with cells growing exponentially

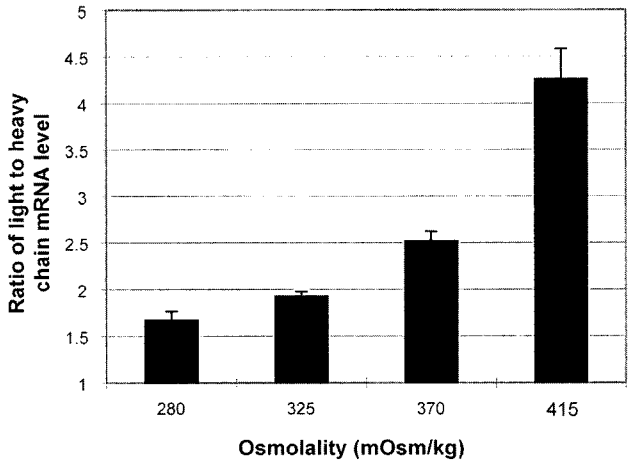

Fig. 6. Effect of hyperosmotic pressure on the ratio of light to heavy chain mRNAs. The error bar represents standard deviation for n=3.

at various osmolalities. The amount of Ig polypeptides synthesized at the end of 30-min pulse time was measured. The net rate of intracellular accumulation of Ig polypeptides equals the translation rate minus the sum of the decay rate, secretion rate, and dilution rate by growth. For a 30-min pulse time, no significant Ig polypeptides were detected in the extracellular media. Further, a pulse-chase experiment revealed that intracellular degradation of Ig polypeptides for a 30-min pulse was insignificant (data not shown). In addition, because a pulse time was much shorter than the doubling time of the cells, the cell number did not change either. Accordingly, the net rate of intracellular accumulation of Ig polypeptides approximately equals the translation rate.

As shown in Fig. 7, both heavy and light chain-specific translation rates increased at elevated osmolalities, but to a

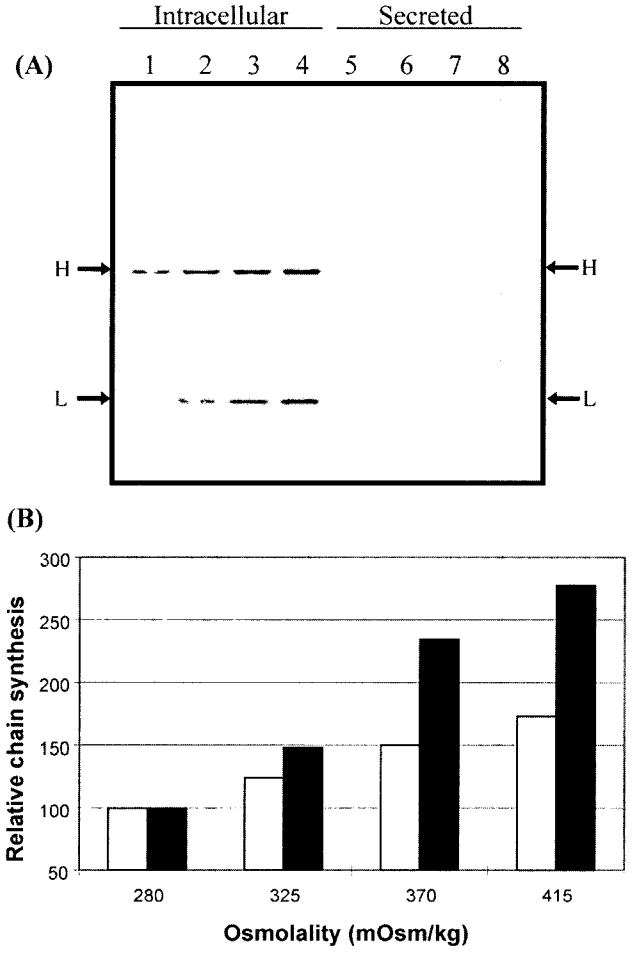

Fig. 7. Effect of hyperosmotic pressure on translation rates of heavy and light chain polypeptides.
(A) H69K-NGD cells were labeled with [^{35}S]methionine for 30 min, and intracellular and secreted antibodies were immunoprecipitated. Incorporation of [^{35}S] methionine at 280 mOsm/kg (lanes 1 and 5), 325 mOsm/kg (lanes 2 and 6), 370 mOsm/kg (lanes 3 and 7), and 415 mOsm/kg (lanes 4 and 8). (B) Relative translation rates of heavy (■) and light (□) chains at indicated osmolalities. The band intensity at the indicated osmolalities was normalized by that at 280 mOsm/kg.

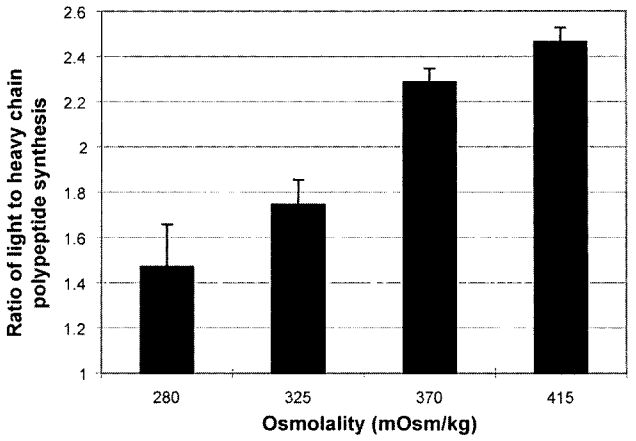

Fig. 8. Effect of hyperosmotic pressure on the ratio of light to heavy chain translation rate. The error bar represents standard deviation for n=3.

different degree. The light chain-specific translation rate at 415 mOsm/kg increased by approximately 180%, compared to that at 280 mOsm/kg. On the other hand, the heavy chain-specific translation rate at 415 mOsm/kg increased by approximately 70%, compared to that at 280 mOsm/kg. The enhancement of heavy and light chain-specific translation rates induced by hyperosmotic pressure was more significant than that of other intracellular proteins. The translation rate of total protein at 415 mOsm/kg, which was determined by total TCA-precipitable cpm per cell after 30-min label with [^{35}S]methionine, increased by approximately 33%, compared to that at 280 mOsm/kg (data not shown).

The ratio of the light chain-specific translation rate to heavy chain-specific translation rate was calculated by correcting the ratio of the respective signals of polypeptides for the proportion of methionine in each polypeptide. This ratio was found to increase from 1.5 to 2.5 as medium osmolality was elevated from 280 to 415 mOsm/kg (Fig. 8).

DISCUSSION

Hyperosmotic pressure increases the q_{Ab} of antibody-producing cells, although it depresses cell growth. While much work has been performed on the effect of hyperosmotic pressure to antibody production and cell growth, there are few reported studies to date, except for our study with KR12H-2 tranfectoma [11], on the intracellular responses of antibody synthesis of transfectoma to hyperosmotic pressure. It has been suggested that enhanced q_{Ab} of KR12H-2 tranfectoma at hyperosmolalities is due to elevated transcription rates of Ig mRNAs and expedited post-translational processing of Ig, and that antibody secretion by KR12H-2 tranfectoma is most likely controlled at the level of heavy-chain translation [11]. In the present study,

we investigated a series of intracellular events of antibody synthesis of H69K-NGD transfectoma at hyperosmolalities in an effort to improve recombinant antibody production.

When subjected to hyperosmotic pressure, H69K-NGD transfectoma displayed decreased μ and increased q_{Ab}. Dry cell weight increased at higher osmolality, in which both protein and nonprotein components were evenly increased. This increased cell mass may influence the way that q_{Ab} is interpreted, because q_{Ab} is calculated based on cell numbers, not based on cell mass. However, increased cell mass at higher osmolality was not primarily responsible for enhanced q_{Ab}, because the enhancement of q_{Ab} was much more significant than that of cell mass.

Hyperosmotic pressure was found to increase the total RNA content of H69K-NGD transfectoma. As observed in the case of butyrate treatment [20, 21], it may induce transcriptional activation due to the change in chromatin structure. Under hyperosmotic pressure, unbinding of histones from DNA results in the dispersal of chromatin. These exposed segments of the chromatin structure are more accessible to RNA polymerase for mRNA transcription [22]. However, under hyperosmotic pressure, light chain-specific mRNA per cell was not enhanced as much as q_{Ab}. Furthermore, heavy-chain mRNA per cell was not enhanced with increasing osmolality. Thus, unlike KR12H-2 tranfectoma, enhanced q_{Ab} of H69K-NGD transfectoma at hyperosmolalities was not due to elevated levels of Ig mRNAs, and the intracellular response of transfectomas to hyperosmotic pressure is cell-line dependent.

Ribosomal RNA constitutes about 80% of the total cellular RNA, and the number of rRNA present on each mRNA plays an important role in translation. Hence, it can be inferred that greater availability of rRNA in cells under hyperosmotic pressure resulted in higher Ig translation rates. As observed in mouse plasmacytoma MPC-11 cells [13], the synthesis of the Ig polypeptides in H69K-NGD transfectoma may be significantly more resistant than the synthesis of other cellular proteins to hyperosmotic pressure. It was also found that the enhancement of heavy and light chain-specific translation rates induced by hyperosmotic pressure was more significant than that of other intracellular proteins. Accordingly, hyperosmotic pressure appeared to preferentially enhance Ig translation rates of H69K-NGD transfectoma. It is also noteworthy that hyperosmotic pressure increased the light-chain translation rate more than the heavy-chain translation rate. However, under hyperosmotic pressure, the translation rate of Ig polypeptides was not enhanced as much as q_{Ab}. This result suggests that hyperosmotic pressure also influences the post-translational process. This understanding of enhanced q_{Ab} of H69K-NGD transfectoma at hyperosmolalities at the basic cellular levels may eventually lead to a better insight into possible environmental or genetic manipulation approaches for improving q_{Ab} of transfectoma.

Acknowledgments

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