Elevation of hepatic sulphotransferase activities in mice with resistance to cystic fibrosis

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The severity of intestinal disease in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) (−/−) mice has been reported to co-segregate with gene loci which contain the genes for hydroxysteroid sulphotransferase (SULT). Because of the potential involvement of steroid hormones in CF, we investigated levels of steroid SULT activity in the livers of CFTR mice to determine whether the levels of SULT activity correlate with the occurrence or severity of CF. To elucidate the possible role of SULT activity in ameliorating the deleterious effects of CF in CFTR (−/−) mice, we determined the levels of phenol SULT (PST), hydroxysteroid SULT [dehydroepiandrosterone (DHEA)-ST] and oestrogen SULT (EST) activity in control CFTR (+/+), heterozygous CFTR (+/−) and homozygous CFTR (−/−) mice, which survive to adulthood. The level of PST activity was not significantly different between any of the groups of mice, regardless of sex or genotype. Although DHEA-ST activity was significantly higher in female mice than in male mice, there was no difference in DHEA-ST activity that could be correlated with genotype. In contrast with PST and DHEA-ST activities, we found that some male and all female adult CFTR (−/−) mice had elevated, dramatically different levels of EST from both CFTR (+/+) and CFTR (+/−) mice. Results from these SULT activity experiments were confirmed by Northern-blot analysis of mouse-liver RNA. Subsequent studies with preweanling mice revealed no differences in the levels of EST that could be correlated with genotype. Thus this study indicates that EST is elevated significantly in CFTR (−/−) mice which survive to adulthood and provides important biochemical information that EST levels may be protective in CF.

Key words: cystic fibrosis transmembrane conductance regulator, β-oestradiol, knock-out mice, dehydroepiandrosterone.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by disruption of the gene encoding the CF transmembrane conductance regulator (CFTR) [1,2]. The CFTR plays a crucial role in Cl^- secretion; absent or diminished CFTR function confers a phenotypic deficit in this Cl^- secretion, resulting in the clinical manifestations of the disorder. To elucidate the role of the CFTR in CF and, in particular, its role in the pathway for Cl^- secretion, a CF knock-out mouse model system has been developed in which homologous disruption of the murine gene encoding the CFTR occurs [3]. Mice without CFTR and with disease-associated mutations can provide a mechanistic framework with which we could study CF disease mechanisms, genotype-based therapeutic approaches and pharmaceutical interventions.

Within the murine CFTR (−/−) genotype, Rozmahel et al. [4] have defined three classes of CF mice (Classes I–III) based on the survival phenotype. Class I mice are those CFTR (−/−) mice which die within 10 days of birth, whereas Class II mice die at or slightly after the time of weaning, approx. 6 weeks after birth. However, some Class II mice live to adulthood if maintained on a liquid diet. Unlike other CF mice, Class III mice live well beyond weaning into adulthood, eating a normal diet. Strain-specific differences in survival suggest that the modulation of disease severity is determined genetically [4]. As detected by genetic analysis, a locus on mouse chromosome 7 is associated with this increase in survival and is believed to encode a modifier for severity of CF, although the nature of this modifier is yet to be determined. Identification of this modifier gene(s) would be a significant step in understanding the secondary genetic factors associated with CF, which might confer prolonged survival to those animals afflicted with the disease. The proximal region of the mouse chromosome 7 to which genetic-linkage analysis maps this modifier has a conserved synteny with human chromosome 19q13. Because murine hydroxysteroid sulphotransferase (SULT) genes map to this modifier locus on chromosome 7 and the human hydroxysteroid SULT gene family maps to a corresponding locus in humans which protects against CF-related meconium lilius [5,6], we have hypothesized that this locus encodes a genetic modifier associated with CF.

In humans, three hydroxysteroid SULT genes have been identified at locus 19q13 [5,6]. In mice, the number of hydroxysteroid SULT genes expressed in mouse liver is not known. However, from the standpoint of biochemical SULT detection, dehydroepiandrosterone (DHEA) can be used as a selective substrate for assaying hydroxysteroid SULT activity, whereas 4-nitrophenol and β-oestradiol (E2) can be used selectively to assay phenol SULT (PST) and oestrogen SULT (EST) activities in mouse liver, respectively [7,8].

DHEA-SULT (DHEA-ST), as well as EST, catalyses the sulphoconjugation of steroid hormones (hydroxysteroids and oestrogens, respectively) at the 3-hydroxyl position [9]. Sulphated hormones lose their ability to interact with their specific cytoplasmic receptors and are therefore unable to initiate subsequent transcriptional effects. However, sulphation also renders steroid hormones more soluble and thus has a potential role in facilitating hormone transport via the plasma. The hormones may then be desulphated at the target tissue before exerting a physiological

Abbreviations used: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; DHEA, dehydroepiandrosterone; SULT, sulphotransferase; DHEA-ST, dehydroepiandrosterone-SULT; EST, oestrogen SULT; E2, β-oestradiol; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; PNP, *p*-nitrophenol; PST, phenol SULT.

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effect [10]. It has long been suspected that steroid hormones play a role in CF severity because male and female CF patients have dissimilarities in disease progression. Like other metabolic enzymes, the SULTs are expressed in the liver, where they are believed to play a role in systemic-hormone homoeostasis. Because of the potential involvement of steroid hormones in CF pathogenesis, and because of the identification of a CF modifier mapping to a SULT gene locus and the role of SULT activity in the regulation of steroid hormones, we investigated the levels of SULT activity in the livers of CFTR mice to determine whether there is any correlation between the levels of SULT activity and occurrence or severity of CF. To elucidate the roles of DHEA-ST, EST and potentially other SULTs in ameliorating the deleterious effects of CF disease in CFTR (−/−) mice, we determined the levels of activity of DHEA-ST, EST and PST activity in control CFTR (+/+), heterozygous CFTR (+/−) and homozygous CFTR (−/−) mice.

MATERIALS AND METHODS

Materials

C57BL/6.Cftrtm/Kth mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). *p*-Nitrophenol (PNP), E2 and DHEA were obtained from Sigma (St. Louis, MO, U.S.A.). [^{32}P]dCTP (3000 Ci/mmol), [^{3}H]E2 (56 Ci/mmol), [^{3}H] DHEA (79 Ci/mmol) and [^{35}S]3′-phosphoadenosine 5′-phosphosulphate (PAPS) (2 mCi/mmol) were purchased from DuPont–NEN (Boston, MA, U.S.A.). PAPS was purchased from Dr Sanford Singer (University of Dayton, Dayton, OH, U.S.A.). RNA STAT-60 was purchased from Tel-Test (Friendswood, TX, U.S.A.). Primers were synthesized by the Molecular Biology Core Facility of the Comprehensive Cancer Center at UAB. Quickhyb was obtained from Stratagene (La Jolla, CA, U.S.A.). All other chemicals were of reagent grade quality.

CF mice

Congenic C57BL/6.Cftrtm/Kth mice (Jackson Laboratory) were maintained on a liquid diet in the Cystic Fibrosis Center at UAB. Animals were bred to produce mice of different CFTR genotypes [homozygous dominant (+/+), heterozygous (+/−), homozygous recessive (−/−)]. Genotypes were confirmed by PCR analysis using primers and protocols obtained from the Jackson Laboratory. A common primer 5′-TTCAAGCCCAAGCTTTCGCGAG was used with a second primer 5′-CTCCCTTCTTCTAGTCACAACCG to generate a 300 bp product from the mutant-CFTR allele. The common primer was used with a third primer 5′-CATCTTGATAGAGCCACGGTGC, to generate a 430 bp product from the wild-type allele. For removal of livers, mice were killed using ketamine and the livers were excised, weighed, immediately frozen in liquid nitrogen and stored at −70 °C until analysis. In the initial study, we analysed livers from both male and female adult mice for each CFTR genotype (+/+, +/−, −/−), resulting in a total of six different groups, with 11 or 12 mice per group. All adult mice were more than 20 weeks old. Subsequent to this initial study, all evaluations were repeated using young mice killed immediately before weaning.

Liver cytosolic SULT activities

To prepare liver cytosols, approximately half of each frozen mouse liver was weighed and homogenized on ice in 5 vols. of 10 mM phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 10% glycerol. Liver homogenates were centrifuged at 100000 ***g*** for 1 h to prepare cytosol. The level of protein in each supernatant fraction (cytosol) was determined by the method of Bradford et al. [11]. Subsequently, the level of EST, PST and DHEA-ST activity in each liver cytosol was determined using a specific substrate for each SULT activity.

EST activity was assayed using 20 nM [^{3}H]E2 as a substrate, with PAPS (10 μM) as the sulphonate donor in an alkaline-chloroform extraction protocol as described previously [12]. At this concentration, E2 is a selective substrate for EST [6]. DHEA sulphation activity was assayed in the same manner but with 10 μM DHEA as substrate [13]. PST activity was assayed with 4 μM PNP which is a selective substrate for PST activity and 20 μM [^{35}S]PAPS using the barium precipitation procedure [14]. All SULT reactions also contained 10 mM sodium phosphate, pH 7.4, and 10 mM $MgCl_2$ in a final volume of 0.125 ml.

Differences in SULT activity were analysed initially by one-way Anova with $P < 0.05$ being considered significant. If large differences were detected, the activities between individual groups were compared using the Student's *t* test with a significance value of $P < 0.05$. Pearson correlation was used to evaluate significance of EST activity to body weights of the different groups of mice. Statistical analyses were carried out using the SPSS Base 10 programs.

Northern-blot analysis of liver EST mRNA

For Northern-blot analysis, primers were prepared using the mouse EST sequence as described by Song et al. [8]. Using the forward primer 5′-TCCGTGGAGTTCTAATGGACAAAC and the reverse primer 5′-ATTTCAGACACAAGGAAGTGGCTC, an 867 bp PCR product was generated from mouse total RNA. For use as a probe, this PCR product was gel-purified and radiolabelled with [^{32}P]dCTP using the Prime-It Random Priming Kit (Promega).

Total RNA was prepared from the remaining half of each liver using RNA STAT-60, as described by the manufacturer (Tel-Test). RNA from each liver was resuspended in 200 μl of diethyl pyrocarbonate ('DEPC')-treated water and then quantified by absorbance at 260 nm. For each liver, 500 μg of total RNA was used to isolate poly(A^+) RNA (polyadenylated) using the Oligotex mRNA mini kit (Qiagen, Valencia, CA, U.S.A.). Subsequent to quantification by absorbance at 260 nm, formaldehyde–agarose gel electrophoresis was performed with 2 μg of poly(A^+) RNA from each liver. RNA was then transferred to a Magnagraph nylon membrane (MSI) using a Turbo blotter (Schleicher and Scheull), UV cross-linked and probed for EST mRNA using Quickhyb (Stratagene). After visualization of the blot by autoradiography, a mouse β-actin-oligonucleotide probe (Clontech) was radiolabelled and used to probe the same blot to confirm that equivalent amounts of RNA from each liver were loaded on the gel.

RESULTS

CF mice

In CF mice, the homozygous disruption of the murine gene encoding the CFTR typically results in death 2–4 weeks after birth, although a subset of mice survive to adulthood, if maintained on a liquid diet. As shown in Table 1, we found that both male and female adult CF mice were of significantly lower body weights than either wild-type or heterozygous mice. To gain additional information in interpreting results of liver ST levels, each mouse liver was weighed at the time of killing. Table 1 shows that for both male and female adult mice, CFTR (+/+) and CFTR (+/−) livers did not differ significantly in weight. For both male and female mice livers in the CF mice (CFTR

Table 1 Comparison of total body weight and liver weight for adult male and female mice of different CFTR genotypes

For each mouse, total body weight and liver weight were determined at the time of killing. Each group consisted of 11 or 12 mice. To determine the percentage of liver weight to total body weight, the liver weight was divided by total body weight and multiplied by 100 (%). Statistical analysis was performed with the Student's *t* test by comparing the (+/−) and (−/−) genotypes with the (+/+) genotype for each sex.

	Male mice			Female mice		
Genotype	Body weight (g)	Liver weight (g)	%	Body weight (g)	Liver weight (g)	%
+/+	33.0 ± 4.2	1.62 ± 0.25	4.9	24.3 ± 2.3	1.19 ± 0.16	4.9
+/−	33.4 ± 3.3	1.56 ± 0.34	4.7	26.2 ± 2.4	1.20 ± 0.16	4.6
−/−	23.6 ± 5.6*	1.15 ± 0.29*	4.9	19.3 ± 2.8*	0.90 ± 0.16*	4.7

* $P < 0.005$.

Table 2 Sulphation of PNP and DHEA by adult male and female mouse liver cytosols from different CFTR genotypes

Cytosol prepared from each mouse liver was assayed for PNP- and DHEA-sulphation activity. Results are expressed in pmol sulphated product generated per mg cytosolic protein per min. The value shown for each group of mice is the mean value for 11 or 12 mice. There were no significant differences in PNP or DHEA sulphation activities between the CFTR genotypes for both male and female mice.

	Male mice		Female mice	
CFTR genotype	PNP	DHEA	PNP	DHEA
+/+	16.32 ± 1.57	0.29 ± 0.08	16.13 ± 0.92	5.37 ± 2.18
+/−	15.66 ± 0.82	0.26 ± 0.11	16.54 ± 1.44	7.24 ± 3.80
−/−	16.23 ± 2.00	0.29 ± 0.09	17.48 ± 1.71	6.47 ± 2.10

Table 3 Sulphation of PNP and DHEA by young, preweanling male and female mouse liver cytosols from different CFTR genotypes

Cytosol prepared from each mouse liver was assayed for PNP- and DHEA-sulphation activity. Results are expressed in pmol sulphated product generated per mg cytosolic protein per min. The value shown for each group of mice is the mean value for 11 or 12 mice. There were no significant differences in PNP- or DHEA-sulphation activities between the CFTR genotypes for either male or female mice.

	Male mice		Female mice	
CFTR genotype	PNP	DHEA	PNP	DHEA
+/+	15.32 ± 1.42	55.6 ± 5.9	16.10 ± 2.72	49.1 ± 10.7
+/−	14.86 ± 0.92	60.5 ± 6.7	16.59 ± 2.14	48.7 ± 8.4
−/−	15.81 ± 2.73	53.0 ± 5.6	17.61 ± 1.91	44.1 ± 12.9

−/−) weighed significantly less than the CFTR (+/+) and CFTR (−/−) livers. However, the lower liver weights in the CF mice correlate with the overall, lower body weight of these animals. Thus the percentage of liver weight to body weight is consistent in all groups of mice.

Liver cytosolic SULT activities

SULT activities were assayed in the adult CF mice to determine whether changes in steroid sulphation activity were associated with augmented survival. Results from DHEA-ST and PNP-ST assays with adult mouse liver cytosol, reflecting liver levels of hydroxysteroid SULT and PST, respectively are shown in Table 2. The level of PST activity was not significantly different between any of the groups of mice, regardless of sex and genotype. Although DHEA-ST activity was significantly higher in female mice than in male mice, there was no difference in DHEA-ST activity that could be correlated with genotype. DHEA-ST activity did not differ significantly between the different genotypes of either female or male mice. DHEA-ST normally shows sexually dimorphic levels of activity with females having approx. 20-fold higher levels than with males [15,16].

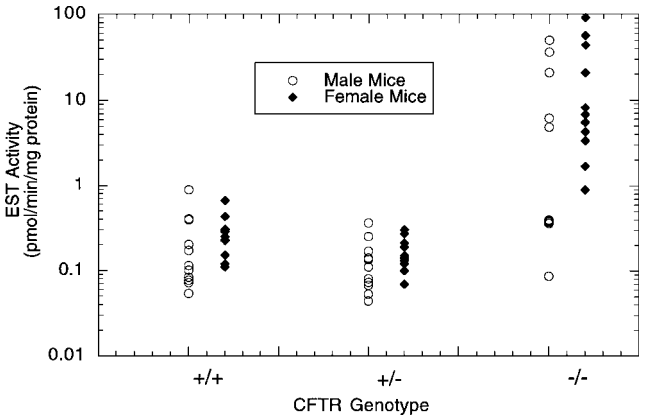

Figure 1 Sulphation of E2 by adult male and female mouse liver cytosols of different CFTR genotypes

Cytosol prepared from each mouse liver was assayed for EST activity using 20 nM E2 as substrate. Because of the variability of sulphation activities within each group of mice, results are presented as a scatter graph rather than as a table. Each dot represents an individual mouse, with 11 or 12 mice per group. Results are expressed in pmol sulphated product generated per mg cytosolic protein per min. The EST activity of the male and female mice of a given genotype was not significantly different. The EST activity of both the male and female (+/+) mice was significantly different from the activity of the (+/−) mice. The EST activity of the male (−/−) mice was significantly different from both the male (+/+, $P < 0.001$) and (+/−, $P < 0.001$) mice. The EST activity of the female (−/−) mice was significantly different from both the female (+/+, $P < 0.01$) and (+/−, $P < 0.01$) mice.

Table 3 presents results from the same analyses performed with young, preweanling mice. Although PST activity was slightly higher in female when compared with male mice and DHEA-ST activity was lower in female mice, there was no correlation of SULT activities with genotype. Thus for female mice, all genotypes had the same level of PST activity and the same level of DHEA-ST activity. A similar result was observed with male mice.

Figure 1 presents the results of liver cytosolic EST activity assays from adult mice. Within each group of mice, there was a significant degree of variation in EST liver activity. Thus these data are presented as scatter graphs rather than in tabular form. The greatest degree of EST variation occurred within both the male and female CFTR (−/−) mice. Despite the amount of variation within these groups, EST levels were elevated markedly in the homozygous CFTR mice as compared with either the wild-type or the heterozygous CFTR mice. In many of the CFTR (−/−) mice, EST levels were orders of magnitude higher than in wild-type (+/+) or CFTR (+/−) mice. All of the female CFTR (−/−) mice had EST levels greater than the levels in the (+/+) or (+/−) mice. The male CFTR (−/−) mice were separated into two groups: one group with elevated EST levels and the other group with apparently normal levels.

Figure 2 presents results of the analogous study examining SULT activities using preweanling mice. There is a large degree of variation within each group. For the male mice, there is no

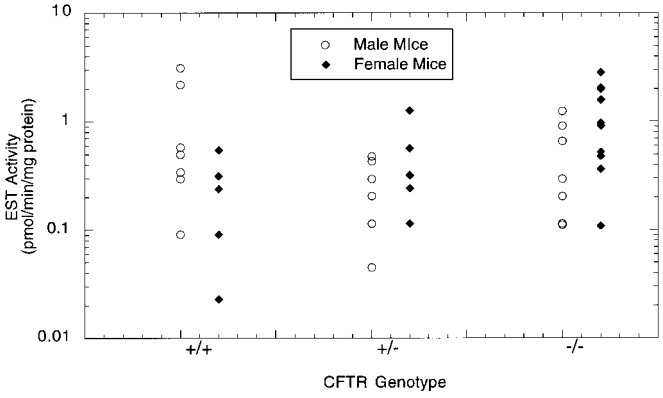

Figure 2 Sulphation of E2 by young, preweanling male and female mouse liver cytosols of different CFTR genotypes

Cytosol prepared from each mouse liver was assayed for EST activity using 20 nM E2 as substrate. Each dot represents an individual mouse. No significant differences were observed in EST activities between the male mice. Between the female mice, only the (−/−) mice were significantly different from the (+/+) mice, $P < 0.02$.

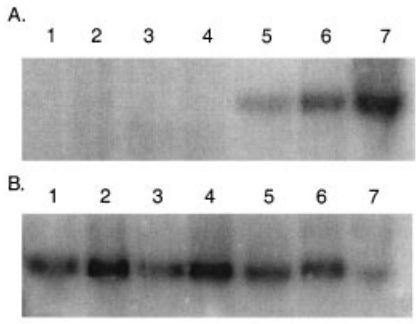

Figure 3 Northern-blot analysis of hepatic EST-message levels in mice with different levels of EST activity

To confirm that E2 cytosolic sulphation levels correlated with EST enzyme, Northern-blot analysis of liver poly(A^+) RNA was performed with a mouse EST cDNA (**A**) or β-actin (**B**) as probes. Results from representative male and female mice from each CFTR genotype with a range of E2 sulphation activities (pmol sulphated product per min per mg protein) are shown. Lane 1, male CFTR (+/+), EST 0.172; lane 2, female CFTR (+/+), EST 0.224; lane 3, male CFTR (+/−), EST 0.140; lane 4, female CFTR (+/−), EST 0.270; lane 5, male CFTR (−/−), EST 21.000; lane 6, female CFTR (−/−), EST 57.024; lane 7, female CFTR (−/−), EST 92.345.

significant difference in EST levels between the three genotypes. For female mice, there is no significant difference between CFTR (+/+) and (+/−) mice, but the EST levels for the (−/−) mice are slightly but significantly higher than those of the other genotypes.

Northern-blot analysis of liver EST levels

To confirm that the elevated EST activity levels found in CFTR (−/−) mouse livers were a result of elevated levels of mouse EST expression, Northern-blot analysis of mouse liver RNA was performed using a cDNA probe, prepared using primers for the published mouse EST sequence [17]. Figure 3 presents a Northern blot with poly(A^+) RNA from representative mouse livers with a range of EST activity levels. Northern-blot analysis indicates that livers with elevated cytosolic EST activity also have elevated EST mRNA levels (A) and that the EST mRNA level for a

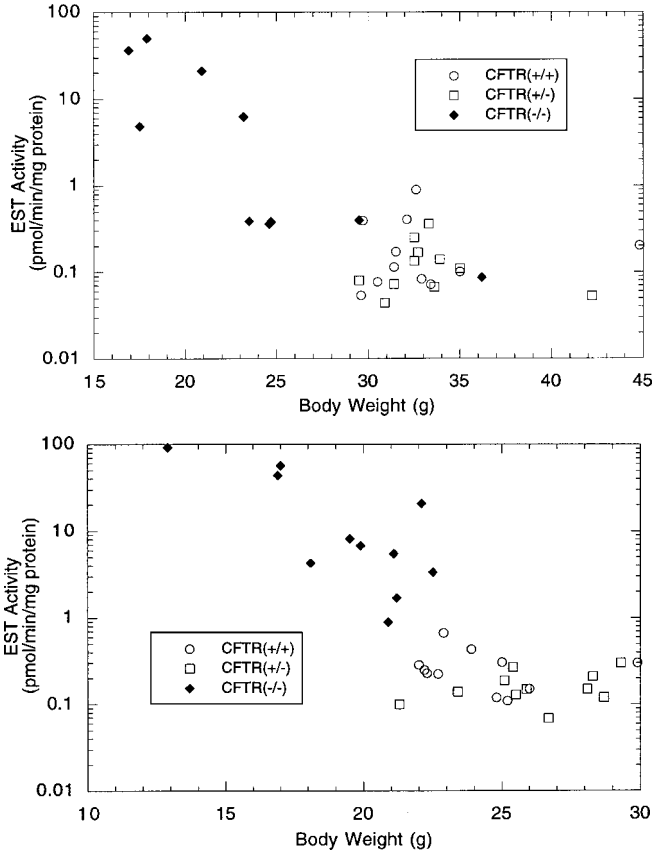

Figure 4 Total body weight was compared with liver cytosolic EST activity for both male and female adult mice

The results for male mice are shown in (**A**) and for female mice in (**B**). For male mice, there was a correlation of −0.62 between body weight and EST activity; for female mice, this correlation was −0.87. The correlations were significant at the 0.01 level.

particular liver correlates with its EST activity level. Thus the high EST activity levels found in the CFTR (−/−) livers are in fact associated with increased expression of EST message.

Comparison of EST activity with body weight

A comparison of total body weight with liver cytosolic EST activity is shown in Figure 4, for both male and female adult mice. This comparison was made to ascertain whether there is a correlation between total body weight, genotype and EST activity. It is apparent that CFTR (+/+) and CFTR (+/−) mice, within each sex, have similar body weights and EST levels. There was a significant negative correlation between EST activity and body weight in both male and female (−/−) mice. For male CFTR (−/−) mice (Figure 4A), the five smallest mice had the highest EST activity levels; the other mice in this group had EST levels that appear to be in the normal range. For female CFTR (−/−) mice (Figure 3B), EST levels were elevated in all of the mice, with a wide range of these elevated levels (0.90–97.00 pmol·min^{-1}·mg protein^{-1}). Additionally, the larger female CFTR (−/−) mice were of weights similar to several of the CFTR (+/+) and CFTR (+/−) mice, but still had much higher EST activity levels.

DISCUSSION

Downstream genetic effects have been associated with loss of CFTR, e.g. the best characterized consequence of absent CFTR, involves a small (2–3-fold) augmentation of Na^+ channel activity both *in vitro* and *in vivo* [18,21]. Potential modifiers of CF severity, such as altered cytokine levels, altered pulmonary surfactant expression and mannose-binding protein defects, exhibit differences of only a few-fold in CF versus non-CF mouse models or human subjects [22–27]. Moreover, in gene-chip studies, the number of proteins that are up- or down-regulated after CFTR deletion generally show differences of only a few to many fold and are quite limited in number; e.g. 20–30 gene products in lung or liver are consistently modulated by the absent CFTR (R. Rozmahal and J. Whitsett, Paper presented at the VA Cystic Fibrosis Meeting, Williamsburg, June 2001). The finding that EST levels increase by up to 100-fold specifically in surviving (and particularly female) CF mice, together with (1) known differences in survival of male and female CF mice and humans and (2) differences in CFTR bioelectric properties in male and female CF mice, may offer an indication to help determine the relationship between steroid hormone metabolism and disease severity. For example, increased EST activity in the liver and other tissues could alter numerous signalling pathways, including those governing ion-transport mechanisms implicated in the disease.

The major CF modifier locus mapping to mouse chromosome 7 corresponds to the human locus for the human hydroxysteroid SULT gene family. The hydroxysteroid SULT gene loci are located on mouse chromosome 7:4 cM [28]. The EST gene is localized to chromosome 5:44 cM [28]. Because of the physiological role of these enzymes in the metabolism of steroid hormones, we evaluated the steroid sulphation activity in these same mice. We anticipated that DHEA-ST activity might be involved in the modification of the severity of CF and therefore evaluated the levels of these sulphation activities in CF mice. As a control, the level of PST, an unrelated SULT involved in the sulphation of small phenols but not primarily of steroid hormones, was also investigated.

In these experiments we found that levels of DHEA-ST activity between adult male and female mice were different, although no differences in DHEA-ST levels could be correlated with CF genotype. Results from our study show that adult CFTR (−/−) mice have liver cytosolic DHEA-ST levels similar to those in both the control and heterozygous mice. The same situation occurred with preweanling mice. Thus we concluded that liver cytosolic DHEA-ST activity was not correlated with the severity of disease in CF mice. There are, however, other potential mechanisms by which DHEA-ST might confer a protection from the deleterious effects of CF. One possibility is that the CF mice that die at birth (and were not investigated in these studies), have very low levels of DHEA-ST. The levels found in the surviving mice we studied, although sufficiently high to be protective, would not be above the DHEA-ST levels in CFTR (+/−) mice or normal controls. Levels of DHEA-ST in preweanling mice are substantially higher than those found in adult mice. Moreover, in adult mice of all genotypes, DHEA-ST is elevated significantly in female mice as compared with male mice. These considerations would argue against a protective role of liver cytosolic DHEA-ST in CF, since it would be anticipated that female mice, with liver cytosolic DHEA-ST levels approx. 30 times higher than those of male mice, would be less susceptible to CF.

Although we had not anticipated this result, the evaluation of EST levels proved to be much more significant. In contrast with DHEA-ST, we found that some male and all female adult CFTR (−/−) mice had elevated, significantly different, EST levels from either CFTR (+/+) or CFTR (+/−) mice. EST activity in both genders was related to body weight in CFTR (−/−) mice. In particular, the smallest mice in each group had the highest EST levels. However, larger mice in the CFTR (−/−) group reached body weights that overlapped the weights of CFTR (+/+) and CFTR (−/−) mice. For female mice, these CFTR (−/−) mice in the same weight range as CFTR (+/+) and CFTR (+/−) mice had comparatively much higher EST levels, indicating that EST level is not a direct result of body weight. Within the CFTR (−/−) group, mice with the highest EST activities had smaller body weights. The possibility that EST levels are generally related to the overall health in mice may be considered, but evidence against this exists in the case of obese diabetic mice, where the larger mice have elevated EST levels. In *db*/*db* and *ob*/*ob* C57 BL/KsJ mice, there are 10–15-fold increases in EST activity as compared with the smaller, wild-type controls [16].

We have shown in this study that EST, an enzyme closely involved with the metabolism of endogenous oestrogens, is significantly elevated in CFTR (−/−) mice which survive to adulthood. The mechanism by which EST might elicit protective effects in CF is not clear. Singh et al. [2] report that oestrogenic steroids are inhibitors of CFTR-mediated Cl^- secretion across polarized human epithelial cells, but at concentrations higher than those that occur under physiological conditions. Because CFTR (−/−) mice do not possess the CFTR, we have suggested previously that E2, the steroid substrate for EST, might activate Cl^- secretion through non-CFTR-dependent pathways. Preliminary studies in mouse intestine and human intestinal epithelium suggest an E2-dependent mechanism for activating alternative non-CFTR Cl^- secretion in CF mice, which may contribute to the protective effects observed with steroid hormones [29]. Further support for this model exists in studies of the rapid, non-genomic effects of E2 involving a plasma membrane-receptor pathway, triggered by E2 to elicit an acute intracellular Ca^{2+} spike within a few seconds to minutes, in contrast with the longer time course required for nuclear transcriptional effects [30]. In this scenario, higher levels of E2 at the apical membrane would cause increased Cl^- secretion and confer a CF protective effect compensating for lack of CFTR. Although increasing evidence indicates both a direct role for E2 in ion-channel activation and a role in regulating the levels of multiple-ion channels in the plasma membrane, further studies are clearly required to elucidate the relationship between E2 sulphation and epithelial-ion transport *in vivo*.

Interestingly, another aspect of this study involves the difference in EST levels between preweanling and adult mice. With preweanling mice, male mice of all genotypes have similar EST levels, which encompass a wide range of activities within each group. However, adult male CFTR (−/−) have EST levels, which are significantly elevated when compared with CFTR (+/+) or CFTR (+/−) mice. The evaluation of preweanling mice would theoretically include both CFTR mice (which die at weaning) as well as CFTR mice characterized by prolonged survival. We hypothesize that the mice, which die before our evaluation of long-term survivors, are those without elevated EST levels. It has been argued previously that the survivorship in CF mice has a large hereditary component and that those CF mice that die do not possess the protective genetic modifiers. Thus it is interesting to speculate that the population of surviving CFTR (−/−) male mice are those with the ability to elevate EST levels and confer protection from the deleterious effects of CF. The situation with female mice is not as clearly demarcated because preweanling CFTR mice have a mean EST level that is

significantly different from the CFTR (+/+) control EST level. However, the differences in EST levels between CFTR (+/+) and CFTR (−/−) mice are more clearly pronounced in adult female mice than in males. Thus it may be necessary for female CFTR (−/−) mice to possess elevated EST levels at earlier times in their development to confer a survival advantage.

The experiments described here have direct relevance to mRNA surveys intended to identify effects of absent CFTR *in vitro* and *in vivo*. It has been suggested that histologically normal tissues should be used to examine mRNA changes due to absent CFTR. Expressed mRNA sequences in a chronically infected and fibrotic human CF lung would be expected to differ dramatically from a non-CF lung, but the majority of these differences would be attributable to chronic infection and more generic pathways that govern pulmonary scarring and/or inflammation. We observed elevations of EST in livers that were otherwise normal in histological appearance and of proportional size. We also found that EST (but not other SULTs) was increased in CF female mice of the same size as their non-CF littermates. These findings support the specificity of a fundamental EST enzymic abnormality in CF. The differences in EST expression were observed specifically in mice that survived to adulthood and not in preweanling mice or non-CF (+/−, +/+) animals. Therefore it seems probable that elevated EST is tied closely to the CF survival phenotype. Sexual differences in CF are well described in humans, including temporal increases in disease severity and earlier death [31]. The present study indicates that a regulatory pathway, which modifies steroid hormone biosynthesis *in vivo* and influences the intestinal disease, is present in the CF mouse. Since elevated EST is most pronounced in female mice that survive to adulthood, the studies help focus attention on a novel cell-signalling pathway that is likely to confer a survival advantage in CF.

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